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CORRECTION.

The accompanying slip is to replace the title on page 809, Vol. LIV, No. 4, December, 1922. The slip is arranged to be pasted over the place occupied by the title at present.

REDUCTION OF BENZYLIDENE-1-ETHYL-2-DIAZOGLUCONATE.

CORRECTIONS.

On page 19, Vol. LI, No. 1, March, 1922, the § foot-note under Table III should read as follows: Johns, Finks, and Gersdorff (Johns, C. O., Finks, A. J., and Gersdorff, C. E. F., *J. Biol. Chem.*, 1919, xxxvii, 152) obtained 29.50 per cent arginine nitrogen in coconut globulin.

Page 20, the last sentence should read: The protein of the coconut precipitated from the 0.2 per cent sodium hydroxide extract by acidifying with dilute acetic acid shows a similarity in the nitrogen distribution to that of the globulin.

On page 87, Vol. LV, No. 1, January, 1923, Table II, last horizontal column, for *86*, *0.497*, and *4.25* read *95*, *2.661*, and *22.8*.

Page 91, line 11, for *0.497* read *2.661;* line 17, for *4.25* read *22.8*.

LEAD STUDIES.

II. A CRITICAL NOTE ON THE ELECTROLYTIC DETERMINATION OF LEAD IN BIOLOGICAL MATERIAL.

By A. S. MINOT.

(*From the Laboratories of Physiology, Harvard Medical School, Boston.*)

(Received for publication, November 3, 1922.)

In 1919 Denis and Minot (1) published a method for the determination of lead in biological material by means of electrolysis. After ashing the material the lead was extracted with acid and precipitated as sulfide from an ammoniacal solution. The lead was then deposited on the anode from a 10 per cent nitric acid solution of the precipitated sulfides. The deposit of lead dioxide was subsequently treated with potassium iodide and the lead finally determined by titrating the liberated iodine against sodium thiosulfate. This method gave frequent positive results in clinical material. Recently, lead determinations have been made in this laboratory by Fairhall's new chromate method (2) and relatively fewer positive findings have been obtained in clinical material than with the electrolytic method of Denis and Minot. To explain this apparent discrepancy the present critical study of the electrolytic procedure was made.

Denis and Minot precipitate lead as sulfide in a solution alkaline with ammonium hydroxide. Under these conditions three complicating factors are involved:

1. Manganese and iron as well as any other heavy metals present are precipitated as sulfides.

2. Calcium phosphate which is present in considerable amount in the ash of feces or tissues is precipitated when the solution is made alkaline, making in many cases a mass of the consistency of gruel which is exceedingly troublesome to handle.

3. When the solution is made ammoniacal in the presence of phosphates two complicating chemical reactions take place: (*a*) Some or all of the lead is precipitated in the form of phosphate

rather than sulfide and the presence of phosphate interferes with the subsequent electrolysis and cannot be removed without a loss of lead. (*b*) When a solution of manganese salt is made ammoniacal in the presence of phosphates a double manganous ammonium phosphate, $Mn(NH_4)PO_4$, analogous to magnesium ammonium phosphate is formed. A description of this salt may be found in almost any qualitative chemistry.[1] It is extremely insoluble in water and dissolves much less readily in dilute acid than does ordinary manganese phosphate; this being especially true in the presence of ammonium salts. Small amounts of manganese precipitated as sulfide would hardly be carried through the extensive washing with 0.1 N hydrochloric acid, which Denis and Minot use, without being dissolved, but this double phosphate in the presence of ammonium salts might remain undissolved and the manganese might be deposited electrolytically as the dioxide and from this point on give all the reactions of lead dioxide.

Reiman and Minot (4) give figures for manganese in the feces of a normal person. They find as high as 6 mg. of manganese excreted in 24 hours. They also find manganese present in normal bile, which would indicate that it is fairly constantly present in the intestinal contents. The same thing has been repeatedly noted in the course of manganese investigations carried on in this laboratory. This fact at once suggests a possible explanation of the great number of positive lead findings when the electrolytic method of Denis and Minot is used. Meillère (5, 6) has pointed out in a very careful and detailed account of the electrolytic separation of lead from biological material, that manganese must be absent at the time of electrolysis or the results will run high. Neumann (7), Moltke-Hansen (8), and others point out that to make a complete separation of lead from manganese electrolytically, the concentration of nitric acid must be at least 20 per cent. Smith (9) states that manganese can be quantitatively deposited from 3 to 5 per cent nitric acid and that for a complete separation of lead from manganese it is absolutely necessary to have a hot solution, strong current, and 15 to 20 per cent nitric acid.

The conditions under which Denis and Minot deposit lead are by no means ideal for the deposition of manganese (10 per cent

[1] For example, see Treadwell (3).

nitric acid, current of 3.5 to 4.5 amperes, and E. M. F. of 4 to 5 volts). However, the figures in Table I show that from a pure manganese nitrate solution in 10 per cent nitric acid electrolyzed under the conditions maintained by Denis and Minot, some manganese is deposited.

While it is true that these actual amounts of manganese are small, there is a readily visible deposit which resembles lead peroxide in appearance, and significant amounts of the thiosulfate solution are used in titrating the liberated iodine. These amounts,

TABLE I.

Electrolytic Deposition of Manganese Dioxide from Solutions of Pure Manganese Nitrate.

Manganese present.	Volume of electrolytes.	Amperes.	Volts.	Duration of electrolysis.	Thiosulfate used.	Manganese equivalent.	Lead equivalent.	Remarks
				Platinum gauze anode.				
mg.	*cc.*			*min.*	*cc.*	*mg.*	*mg.*	
5.0	60.0	3.0	4.5	30	0.55	0.08	0.30	Copper spiral cathode.
2.0	60.0	3.0	4.5	30	1.35	0.19	0.74	" " "
2.0	60.0	1.2	4.5	30	1.20	0.16	0.65	" " "
				Small platinum crucible anode.				
5.0	6.0	3.0	4.5	30	2.20	0.31	1.20	Copper spiral cathode.
2.0	6.0	1.2	4.5	30	1.82	0.25	1.00	" " "

erroneously calculated as the equivalent amount of lead, would give as much as is often found in clinical tests.

Table II shows recoveries of lead from pure lead solutions both in small volume with high current as Denis and Minot used, and from a larger volume with somewhat lower current run for a longer time, as we have preferred to use in this laboratory.

Table III shows the electrolysis of lead in the presence of varying amounts of manganese—showing invariably too high results due to a deposition of manganese dioxide with the lead peroxide on the anode.

These results indicate clearly that over a considerable range of conditions under which lead can be quantitatively deposited, manganese is also deposited in amounts great enough to vitiate entirely quantitative results when lead is present or to give an apparent qualitative test for lead when no lead is present.

TABLE II.

Recovery of Lead from Lead Nitrate Solution by Electrolysis.

Concentration of nitric acid.	Volume of electrolytes.	Amperes.	Volts.	Duration of electrolysis.	Lead found.	Lead present.	Remarks.
Platinum dish as anode.							
per cent	*cc.*			*min.*	*mg.*	*mg.*	
20	20	6.5	5.0	20	1.02	1.00	Copper spiral cathode.
20	20	6.5	3.0	20	0.95	1.00	" " "
20	20	6 5	3.2	20	0.91	1.00	" " "
10	20	6.5	4.3	20	1.09	1.00	" " "
12	20	6.5	4.5	20	0.95	1.00	" " "
Platinum gauze anode.							
10	60	3.5	4.0	45	5.06	5.00	Copper spiral cathode.
10	60	3.5	10.0	30	1.94	2.00	" " "
10	60	3.5	10.0	45	1.90	2.00	" " "
8	60	3.0	10.5	70	1.95	2.00	" " "
Small platinum crucible anode.							
10	6	3.0	10	30	1.98	2.00	Copper spiral cathode.
Platinum gauze anode.							
10	60	1.2	4.5	90	1.98	2.00	Copper spiral cathode.

Table IV shows unselected results from the analysis of ten specimens of normal feces. These specimens were carried through the entire procedure exactly as described by Denis and Minot. In some cases the precipitated sulfides were washed many times more than was apparently necessary to see whether the manganese could not be all removed. In eight out of ten cases a significant

TABLE III.

Recoveries of Lead from Mixtures of Lead and Manganese Nitrates by Electrolysis.

Concentration of nitric acid.	Volume of electrolytes.	Amperes.	Volts.	Time.	Apparent lead recovered.	Lead present.	Manganese present.	Remarks.
per cent	*cc.*			*min*	*mg.*	*mg.*	*mg*	
10	60	1.3	5.5	90	1.59	1.00	1.00	Permanganate color during electrolysis.
10	60	1.3	5.5	90	1.62	1.00	1.00	" " " "
10	60	1.3	5.5	90	3.17	1.00	5.00	" " " "
10	60	1.3	5.5	90	5.84	1.00	5.00	" " " "
10	60	1.3	5.5	90	3.17	1.00	2.00	" " " "
10	10	3.5	10.0	20	2.16	1.00	2.00	" " " "
10	10	3.5	10.0	20	2.03	1.00	2.00	" " " "

TABLE IV.

Results of Analysis of Normal Feces by Denis and Minot Method.

No	Amount of feces analyzed.	No of washings with 0 1 N HCl.	Apparent lead found.	Lead determined by chromate method.	Remarks.
	gm.		*mg.*	*mg.*	
1	400	4	2.22	00	Definite brown deposit.
2	200	5	0.60	00	" " "
3	350	5	0.56	00	" " "
4	350	4	0.33	00	" " "
5	350	4	0.55	00	" " "
6	350	5	0.77	00	" " "
7	350	13	0.54	00	" " "
8	350	13	0.00	00	No deposit.
9*	350	6	1.37	00	Distinct deposit.
10	300	6	0.00	00	No deposit.

* 0.29 mg. of lead determined by chromate method in wash acid.

deposit on the anode was obtained. In most cases the electrolytic bath became colored with permanganate color during the electrolysis which showed that manganese was present. The solution of the deposited dioxide was then titrated to find its

lead value, and a lead analysis of the solution was then made by Fairhall's chromate method. None of the patients from whom these specimens were obtained, except Case 9, had any history of exposure to lead. Nevertheless, Meillère (10) and others believe that lead is frequently accidentally present in normal tissues and excreta. In no case was lead found in the solution of the deposited dioxide, while in every case a pink permanganate color could be produced. In Case 9, a painter who was suspected of lead poisoning, the acid used for washing the sulfide deposit as well as the electrolytic bath and the solution of the dioxide after titration were analyzed for lead by the chromate method. 0.29 mg. of lead was found in the acid used for washing the precipitate while the other two solutions gave a negative test. Thus, in this case the lead present would have been entirely discarded in the ordinary course of the analysis while in its place manganese dioxide is deposited in sufficient amount to give an apparent lead value of 1.37 mg. though no lead is present in the final solution titrated.

Further studies to try to account for the fairly good recoveries which Denis and Minot report were made. Mixtures of lead and manganese solutions with 0.5 gm. of a mixture of sodium and calcium phosphates to imitate the solution of ash from feces were carried through the entire procedure. The hydrochloric acid solution of these salts was made ammoniacal, and saturated with hydrogen sulfide gas; the precipitated sulfides were removed by centrifugalization, and washed six to eight times with 0.1 N hydrochloric acid. The precipitate remaining after washing was dissolved in concentrated nitric acid, diluted to make a 10 per cent acid solution, and electrolyzed. The acid used for washing the precipitate, as well as the electrolytic bath after electrolysis, and the solution after the titration of the deposited dioxide were then analyzed for lead by Fairhall's chromate method. In this way any loss of lead due to the acid washing or to incomplete electrolytic deposition can be detected and the total lead present quantitatively accounted for. These results are presented in Table V.

In every case the titration of the electrolytic deposit gave a considerably higher value for lead than could be recovered from the titrated solution by the chromate method. This is due to the presence of manganese which is deposited as the dioxide, and often a faint play of pink permanganate color could be seen around the

electrode. In most cases significant amounts of lead, often as much as would be present in clinical material, are dissolved from the sulfide precipitate by the wash acid. There is a further loss owing to incomplete electrolytic deposition because of the interference of phosphate ions which are carried along by the undissolved lead phosphate, and the manganous ammonium phosphate. The sum of the amounts of lead recovered by the chromate method in the electrolytic bath, wash acid, and solution after titration shows good recoveries of the added lead in every

TABLE V.

Comparison of Electrolytic and Chromate Methods in Recovery of Lead from Mixtures of Lead and Manganese Salts.

No.	Lead present.	Manganese present.	Lead calculated from dioxide deposit.	Lead in wash acid.	Lead in electrolytic bath.	Lead after titration.	Total lead recovered by chromate method.	Remarks.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
1	2.0	5.0	1.13	0.47	1.24	0.20	1.91	Electrolyzed 1 hr., 1.5 amperes, and 4.5 to 5.0 volts.
2	2.0	2.0	1.34	0.75	0.97	0.35	2.07	Electrolyzed 1 hr.
3	2.0	5.0	1.73	0.43	1.09	0.68	2.20	" 1½ hrs.
4	5.0	5.0	7.79	0.09	0.12	4.79	5.01	" 1 hr.
5	5.0	5.0	6.11	0.79	0.17	3.91	4.89	" 1 "
6	5.0	5.0	6.04	0.71	0.15	4.05	4.91	" 1 "
7	5.0	5.0	5.82	0.68	0.32	4.02	5.02	" 2 hrs.

case. In the usual electrolytic procedure the loss of lead in wash acid and electrolytic bath is roughly compensated for by deposition of manganese dioxide with the lead dioxide.

Obviously the Denis and Minot method as it stands is decidedly impractical. It is not quantitative, and even less reliable as a qualitative test, because in the analysis of feces the majority of cases where no lead is present give a positive test due to interference of manganese. It could be much improved by precipitating the lead in a faintly acid solution which would hold manganese and phosphate in solution as is shown by Fairhall (2) and many others.

No attempt is made here, however, to work out ideal conditions for deposition of lead electrolytically from solution of the ash from biological material. It is extremely difficult to get rid of the last traces of phosphate in any solution which will also allow a complete precipitation of lead, and even traces of phosphate interfere with the complete deposition of lead as a dioxide. An electrolytic method at best is time-consuming and requires costly apparatus where large numbers of determinations are to be made and we have found the chromate method much more accurate and practical.

SUMMARY.

1. Denis and Minot have described a procedure for the electrolytic determination of lead in which manganese is often present in the electrolytic bath and is deposited with the lead as dioxide on the anode.

2. This method often gives a positive test for lead when no lead is present because manganese dioxide is deposited, which gives the reactions of lead dioxide in the subsequent steps in the procedure.

3. In the Denis and Minot procedure there are losses of lead (*a*) during washing due to solution of the precipitated sulfides, and (*b*) to incomplete electrolytic separation.

4. The errors due to deposition of manganese and loss of lead tend to balance each other and give roughly quantitative results.

5. Fairhall's chromate method has been found more accurate and practical than an electrolytic method, though with some modification the Denis and Minot method could be made satisfactory.

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HYDROLYSIS OF YEAST NUCLEIC ACID WITH DILUTE ALKALI AT ROOM TEMPERATURE. (CONDITIONS OF STEUDEL AND PEISER.)

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 22, 1922.)

In the year 1919, Steudel and Peiser[1] published a communication on the structure of yeast nucleic acid in which they challenged the hypothesis developed in this laboratory. The authors took exception to the tetranucleotide theory, offering in its place a trinucleotide theory. They claimed that the complex yeast nucleic acid was composed of guanine, adenine, and cytosine nucleotides, and further, that the uridine nucleotide present in the commercial product (C. F. Boeringer and Söhne) was an impurity of independent origin. In a later publication (1921) they reported[2] the discovery in commercial nucleic acid, of another impurity; namely, guanylic acid. Finally, in 1922[3] the same authors stated that yeast nucleic acid on treatment with a 3 per cent solution of sodium hydroxide at 15–17°C. liberates from its molecule 1 molecule of guanylic acid, so that the remaining part is completely free from guanine. Steudel and Peiser considered their observation of such importance that they advocated for the present the deferring of the discussion of the structure of yeast nucleic acid.

To the present writer, on the contrary, the observation of Steudel and Peiser seemed to substantiate the theory of the structure of the yeast nucleic acid formulated by him, according to which the individual nucleotides are linked to one another in ester form. A complete analysis of the products formed under the conditions given by Steudel and Peiser confirms still further

[1] Steudel, H., and Peiser, E., *Z. physiol. Chem.*, 1919, cviii, 42.
[2] Steudel, H., and Peiser, E., *Z. physiol. Chem.*, 1921, cxiv, 201.
[3] Steudel, H., and Peiser, E., *Z. physiol. Chem.*, 1922, cxx, 292.

the theory expressed by us, since it was found that simultaneously with the formation of guanylic acid, the other three nucleotides also are formed. Thus all doubts as to the tetranucleotide conception of yeast nucleic acid are removed. The statement of Steudel and Peiser that under their conditions the liberation of guanylic acid is complete was found to be erroneous. Three experiments were performed at three temperatures varying between 15 and 25°C. In each experiment a portion of the nucleic acid remained unchanged. At the highest temperature 12 per cent, at about 20°C., 37 per cent, and at 15°C., 52 per cent remained undecomposed. No attempt was made to regulate the temperature accurately, the variations were caused by weather conditions. The details of the procedure for the separation of the unchanged nucleic acid from the simple nucleotides and for the separation of guanylic acid from the other three nucleotides are given in the experimental part.

EXPERIMENTAL.

Nucleic acid (100 gm.) was dissolved in 400 cc. of a 3.5 per cent solution of sodium hydroxide and allowed to stand at room temperature over night (15 hours). The solution was then separated into three fractions; the first contained unchanged nucleic acid, the second, guanylic acid, and the third, the other three nucleotides.

Nucleic Acid Fraction.—The separation of the unchanged nucleic acid was brought about by precipitation with glacial acetic acid. To test the efficiency of this procedure, purified nucleic acid (40.0 gm.) was dissolved in 130 cc. of water on addition of a little alkali and precipitated with 5 liters of glacial acetic acid. The precipitate was washed with alcohol and ether and dried under diminished pressure over sulfuric acid. The yield of the substance was 39.0 gm. or 97.5 per cent of the original material.

The composition of the two samples was as follows:

	C	H	N	P	Purine bases as hydrochlorides.
					per cent
Original material (No. 101).....	34.79	4.19	15.70	9.05	36
Recovered material (No. 172)....	34.57	4.16	15.58	9.04	38

In the experiments on the action of dilute alkali, 400 cc. of the solution were poured into 20 liters of glacial acetic acid. A precipitate formed, which was dried as described above. The yield of the recovered nucleic acid varied with the room temperature. At 15°C. the yield was 52 per cent, at 20°C., 37 per cent, and at 25°C., 12 per cent. The composition of the recovered material was as follows:

	C	H	N	P	Purine bases as hydrochlorides.
					per cent
From experiment at 15°C. (No. 104)	34.17	4.15	15.62	8.65	39
From experiment at 20°C. (No. 153)	34.31	4.04	15.86	8.84	37

Guanylic Acid Fraction.—The mother liquors from the first fraction were concentrated under diminished pressure (about 10 to 15 mm.) without heating to nearly dryness. The residue was dissolved in about 100 cc. of water (in experiments starting with 100 gm. of nucleic acid) and allowed to stand over night in the refrigerator at a temperature varying from 0 to + 2°C. The acid sodium salt of guanylic acid settled out in the form of a white precipitate and was readily filtered off by suction in the refrigerating chamber. The substance was converted into the lead salt, and this into the free acid. In order to remove all the base the operation had to be repeated three times. Finally, the solution which, on standing over night at 0 to + 2°C., remained perfectly clear, was converted into the brucine salt. After three recrystallizations from 35 per cent alcohol the substance analyzed as follows:

0.1017 gm. substance: 0.1984 gm. CO_2 and 0.0590 gm. H_2O.
0.2000 " " 17.4 cc. N_2, t = 15°C., P = 769.5 mm.
0.3000 " " 0.0274 gm. $Mg_2P_2O_7$.

Brucine salt of guanylic acid (air-dry).
$C_{46}H_{52}N_4O_8 \cdot C_{10}H_{14}N_5 \cdot PO_8 + 7\ H_2O$.

Calculated. C 52.61, H 6.28, N 9.88, P 2.43.
Found. " 53.02, " 6.48, " 10.09, " 2.81.

Fraction Containing Adenine, Cytosine, and Uridine Nucleotides.—The mother liquors from the guanylic acid fractions obtained from the three experiments were combined and converted into the lead salts and these again freed from lead. It was found advisable to repeat this operation several times. The final solution of the nucleotides which reacted acid to Congo red was treated with a methyl alcoholic solution of brucine until the reaction of the original solution turned slightly alkaline. The brucine salts were then fractionated from 35 per cent alcohol exactly in the manner previously described. Three fractions were obtained differing from one another in their solubilities. The most insoluble portion had the properties and the composition of the brucine salt of uridine phosphoric acid. It analyzed as follows:

0.1011 gm. substance: 0.1966 gm. CO_2 and 0.0612 gm. H_2O.
0.2000 " " 12.8 cc. N_2, t = 22°C., P = 755.3 mm.
0.3000 " " 0.0272 gm. $Mg_2P_2O_7$.
Brucine salt of uridine phosphoric acid.
$C_{46}H_{52}N_4O_8 \cdot C_9H_{13}N_2 \cdot PO_9 + 7\ H_2O$.
Calculated. C 53.30, H 6.43, N 6.79, P 2.50.
Found. " 53.02, " 6 77, " 7.43, " 2.53.

The intermediate fraction approached analytically the composition of cytidine phosphoric acid.

0.1011 gm. substance: 0.1594 gm. CO_2 and 0.0588 gm. H_2O.
0.2000 " " 15.4 cc. N_2, t = 22°C., P = 755.3 mm.
0.3000 " " 0.0272 gm. $Mg_2P_2O_7$.
Brucine salt of cytidine phosphoric acid.
$C_{46}H_{52}N_4O_8 \cdot C_9H_{14}N_3 \cdot PO_8 + 7\ H_2O$.
Calculated. C 53.34, H 6.52, N 7.93, P 2.57.
Found. " 52.70, " 6.50, " 8.85, " 2.45.

Finally, the most soluble fraction had the composition of adenosine phosphoric acid.

0.1004 gm. substance: 0.1948 gm. CO_2 and 0.0590 gm. H_2O.
0.2000 " " 17.0 cc. N_2, t = 24°C., P = 766.6 mm.
0.3000 " " 0.0272 gm. $Mg_2P_2O_7$.
Brucine salt of adenosine phosphoric acid.
$C_{46}H_{52}O_8N_4 \cdot C_{10}H_{14}N_5 \cdot PO_7 + 7\ H_2O$.
Calculated. C 53.28, H 6.40, N 10.00, P 2.47.
Found. " 52.92, " 6.57 " 9.86, " 2.53.

No attempt was made to isolate the individual nucleotides in the free state, since the isolation of the brucine salt is sufficient evidence to prove the contention that the bonds between the individual nucleotides of the yeast nucleic acids are very feeble. Thus the theory of an ester linking between the nucleotides is the one which best harmonizes with the experimental facts.

THE CHEMICAL DEFENCE MECHANISM OF THE FOWL.

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Detoxication processes in the organism of the fowl have thus far received but little attention, due no doubt, in part at least, to the disagreeable features of a mixture of urine and feces. The specific detoxicating agent of the fowl is ornithine, and up to the present this substance has been found to combine with but four compounds, namely benzoic acid (1), phenylacetic acid (2), *p*-nitrophenylacetic acid (3), and pyromucic acid, to form the respective detoxication products. Jaffé (4) fed furfural to chickens, in order to study the method employed by them in detoxicating this compound. He found that the furfural was oxidized to the corresponding acid (pyromucic acid), and that 2 molecules of this compound were then combined with ornithine and so excreted. He gave to this product the name of pyromucinornithuric acid. Apparently, therefore, the fowl is unable to make use of the Perkin's reaction, *i.e.* to combine the furfuraldehyde with acetic acid to form furfuracrylic acid, a reaction observed by Jaffé and Cohn (5) in the case of dogs.

Suga's findings, in this connection, are rather remarkable in the light of results obtained by other investigators, inasmuch as he finds (6) that "starving hens (unlike well-fed birds) are unable to synthesize hippuric acid from benzoic acid and glycocoll. They conjugate ingested benzoic acid with ornithine into ornithuric acid and excrete the latter compound." Yoshikawa (7), some 10 years before, investigated this same problem, and decided that chickens were unable to synthesize glycocoll for detoxication purposes as well as to use it if it were supplied to them. He fed birds glycocoll along with benzoic acid, and found only ornithuric acid in the excreta. Hoshiai (8) after feeding pyridine to chickens, found a methyl hydroxypyridine compound in the excreta, showing

that the methylation process is also a possible method of detoxication open to the fowl.

In our work we wished to study in a systematic way the different detoxication processes in the fowl, in order to determine which of the reactions common to other animals were also employed by the fowl. Accordingly, we chose for feeding, such substances as illustrate general classes of reactions, and at the same time are detoxicated in a slightly different manner by different species of animals (9). Under the heading Experimental are given only the observed results, while in the Discussion the fate of each individual compound in the organism of the chicken is compared with that of the same substance in the organism of other animals.

EXPERIMENTAL.

For our work we chose mature fowls, generally cocks, for we found that the adult male birds could withstand the toxic chemicals better than could any of the others. The birds were placed in ordinary metabolism cages made of zinc and so constructed that the bottoms sloped away from the sides toward a funnel-shaped aperture in the center, through which the urine could readily drain. A screen of about $\frac{1}{2}$ or $\frac{3}{4}$ inch mesh formed a floor and thus also prevented the birds from treading upon the excreta. Unless otherwise stated, the birds were fed on a mixed diet of corn, bread, green vegetables, and hemp-seed—the latter being added because of its high content of edestin, and consequently of the amino-acid arginine from which ornithine could readily be derived.

Benzaldehyde.

We fed a large Rhode Island Red rooster of 3.2 kilos body weight, a total of 3 gm. of benzaldehyde, in doses of 1 gm. on each of 3 consecutive days. The substance was fed by means of a stomach tube and washed down with water. No toxic symptoms of any kind were noticed—the bird continuing to eat and drink freely. The drug acted, however, as a diuretic, since more water was drunk than ordinarily and a much more copious excretion of a slimy, mucous-like urine was obtained. The excreta resulting from the 3 days feeding were collected and combined and treated on a water bath, at a temperature of about 75°C., with approximately three times their volume of alcohol. The temperature is worthy of note, since Jaffé in his work with benzoyl ornithine, cautions against allowing the temperature to rise above 40°C. Totani issues a similar warning in his work on phenyl-

acetyl ornithine. However, repeated work with both phenylacetyl ornithine and benzoyl ornithine has failed to demonstrate the necessity of such a precaution. After digesting the excreta for about an hour at the temperature of 75°C., the alcoholic extract was filtered, and the residue extracted again in a similar manner with another triple volume of alcohol The process was then repeated a third time. Finally, the combined alcoholic extract was evaporated on a water bath with the aid of an electric fan. The moist residue thus obtained was placed in a separatory funnel, acidified to Congo red with dilute hydrochloric acid, and extracted three times with ether, using three to five volumes of solvent each time. The combined ether extract was evaporated to about 75 cc. by means of an electric fan. After standing for several days in the ice box, flaky, white crystals appeared. When dried *in vacuo* at 65°C., these crystals melted at 179–181°C. The yield was 0.52 gm.; *i.e.*, about 10 per cent of the theoretical amount. The substance, as evidenced by the analysis (see below), was dibenzoyl ornithine—the same as was found by Jaffé, and which he termed ornithuric acid. On hydrolysis, the substance yielded benzoic acid, as shown by the melting point. According to the Kjeldahl method, the material contained 8.20 per cent nitrogen instead of the theoretical 8.23 per cent, for 0.1251 gm. of substance required 7.40 cc. of 0.1 N acid.

p-Hydroxybenzaldehyde.

As this substance behaves differently in different animal organisms, it was chosen as the second compound for feeding. A large rooster was fed 1.2 gm. of *p*-hydroxybenzaldehyde on each of 3 consecutive days. Small amounts of the material were placed far back on the tongue of the bird and then washed down with water. No untoward symptoms of any kind were observed, as the chicken ate and drank normally immediately after the feeding. A supply of hemp-seed was placed at the disposal of the fowl and was eaten voraciously. The combined excreta were treated the same as in the case of the benzaldehyde feeding, except that the residue from the ether extract was handled somewhat differently. The residue was taken up in a little water and made alkaline with dilute ammonia. To the ammoniacal solution was added 10 per cent calcium chloride solution, in order to remove the fatty acids as the insoluble calcium salt. These fatty acids are ever present in chicken excreta, and as they have much the same solubilities as a number of the compounds we were investigating, they were a constant source of trouble. After the precipitate thus formed was filtered, the filtrate was decolorized with animal charcoal and again filtered, after which it was cooled and acidified to Congo red with dilute hydrochloric acid. Thereupon, there appeared on the sides of the container yellowish red bunches of crystals. After a few days these crystals were filtered off, recrystallized from hot water, and dried at 80°C. *in vacuo*. They then melted at 208–210°C., gave a strong positive Millon's reaction, contained no nitrogen, and were not affected by boiling concentrated acid during a period of 1 hour. The material, therefore, was an hydroxyphenyl com-

pound, but was not p-hydroxybenzaldehyde, for this substance melts at 115–116°C. Moreover, it was not a conjugation product of ornithine, nor of even a non-nitrogenous substance. It could have been only p-hydroxybenzoic acid, which melts at 212–214°C.

Phenylpropionic Acid.

A water solution of the sodium salt of this acid was fed, by means of a stomach tube, to a large Rhode Island Red rooster. The amount administered was 0.8 gm. per day for 3 successive days. It proved entirely non-toxic, and was readily taken by the bird. The 3 days collection of the excreta was handled in exactly the same manner as that described under the treatment of p-hydroxybenzaldehyde.

It was expected that the phenylpropionic acid would undergo β-oxidation, forming finally benzoic acid, with perhaps the formation of β-hydroxy-β-phenylpropionic acid, benzoyl acetic acid, and acetophenone as intermediary products. Accordingly, an attempt was made to determine the presence of these possible intermediary products. This, however, proved impracticable, since the amounts of the substances which might have been formed were so exceedingly small that the presence of fats, fatty acids, and other excretory products, made even the most indefinite qualitative work a decided uncertainty. After working up the excreta, therefore, in the usual way, we obtained about 0.5 gm. of ornithuric acid, along with some free benzoic acid. We did not find even any free phenylpropionic acid. The ornithuric acid, when dried *in vacuo* at 70°C., melted at 179–182°C. A nitrogen determination according to the Kjeldahl method showed 8.16 per cent instead of the theoretical 8.23 per cent.

There was no convincing reason to believe that there were other important compounds in the excreta, except perhaps some hippuric acid, which Suga claims to have found in the excreta of well fed birds. Indeed, on account of the similarity in melting points of the two compounds (hippuric acid, M.P. 187°C., and ornithuric acid, M.P. 182–184°C.), and of their nitrogen contents (hippuric acid, 7.89 per cent N, and ornithuric acid, 8.23 per cent N), one could not conclude definitely either way. We decided, therefore, to test the accuracy of the method for the determination of hippuric acid. To this end we fed a large rooster 1 gm. of hippuric acid on each of 2 consecutive days. The excreta were collected and extracted as usual. As we were able to recover 1.13 gm. of the original material, it seems that our method would have showed, at least qualitatively, the presence of hippuric acid in the former instance had this substance actually been formed in the body of the fowl.

Cinnamic Acid.

As this acid is considerably less toxic than phenylpropionic acid, it was fed in 1 gm. doses at the rate of one dose per day for 3 days. Then, with an intermission of 1 day, a second similar feeding period of 3 days occurred. It was conjectured that the cinnamic acid might be excreted as its sodium

salt, on account of its relatively low toxicity, or that it might combine with ornithine to form the dicinnamoyl ornithine, or that still more probably it would first be saturated to form phenylpropionic acid, then be oxidized to benzoic acid, and be excreted as ornithuric acid.

The compound was fed as a water solution of its ammonium salt, and the excreta were worked up in 3 day collections. The method of working the first collection was exactly the same as that used for the isolation of ornithuric acid. In this way 0.6 to 0.7 gm. of ornithuric acid was obtained—the usual yield; namely, approximately 10 per cent. In working up the second 3 day collection of excreta, we endeavored to determine the amount of free cinnamic acid and dicinnamoyl ornithine which might be present. With this end in view, the final extraction was carried out with ethyl acetate as solvent. The ethyl acetate was then evaporated and the residue taken up with hot water, after which, evaporation was again conducted until crystals appeared on cooling. In this way we obtained about 0.85 gm. of unchanged cinnamic acid from the 3 day collection of excreta after 3 gm. of cinnamic acid had been fed. In order to determine whether any of the original acid had undergone saturation and had been converted thus into phenylpropionic acid, we took the mother liquor from the cinnamic acid crystals, acidified it strongly with phosphoric acid until Congo red showed an excess of mineral acid, and distilled it with steam. This should have carried over any phenylpropionic acid, which is volatile in steam. We were not able to find, however, any trace of phenylpropionic acid.

m-Nitrobenzaldehyde.

A large rooster was fed 1 gm. a day of this substance for 5 consecutive days, while another bird received in the same manner a total of 6 gm. The material proved to be as non-toxic to the chickens as had been found previously for other animals or for human beings. Since the substance is insoluble in water, it was necessary to pulverize it finely, place a pinch of it far back on the bird's tongue, and wash it down with water.

The excreta were collected in the usual manner, dried by means of an electric fan, and then stirred with warm alcohol for 1 or 2 hours. The alcoholic extract was then decanted, and filtered to remove particles of suspended matter. This filtrate was then warmed on a water bath and evaporated to dryness with the aid of a fan. The bulky residue thus obtained, was taken up with just sufficient water to produce a thin, pasty mass, acidified to Congo red with dilute hydrochloric acid, placed in a continuous rotary extractor, and extracted first three times (2 hours each) with 300 cc. of ether, and later, in exactly the same way, with ethyl acetate. In both extracts, but chiefly in the ethyl acetate, there appeared a reddish oil as the solutions were gradually evaporated to dryness. This reddish substance hardened, on exposure to air, into a gummy mass. It was insoluble in water but soluble in practically all the organic solvents, and formed, in boiling water, an unstable emulsion. It was apparently acid in character, for it dissolved readily in dilute alkalies, and precipitated

again upon acidification. In all probability, it was not a complex formed by the conjugation of a benzoic acid derivative with some such compound as ornithine, for the boiling with dilute or concentrated acids under a reflux condenser, which would most certainly have caused hydrolysis of such a compound, effected no perceptible change in the substance. So far, we have not succeeded in obtaining the material in crystalline form. Hence, we are unable to determine its constitution. Still, it is possible to obtain the compound in much greater yields than is the case with any of the other compounds thus far studied in our chicken experimentation.

Nitrobenzene.

Since *m*-nitrobenzaldehyde failed to show the possibility or impossibility of simultaneous oxidation and reduction in the organism of the fowl, as we had hoped it would, we chose two other compounds, which not only occasion this reaction in the bodies of other animals, but are particularly interesting on account of their different methods of detoxication in different species of animals. These compounds were nitrobenzene and *o*-nitrophenylpropiolic acid.

A large Rhode Island Red rooster of 3.1 kilos body weight received 0.5 gm. of nitrobenzene by means of a stomach tube. The material proved to be very toxic, as we had expected. Within a few hours the bird became lethargic, and refused to eat. Within 10 hours the chicken was extremely cyanotic, and showed marked signs of dyspnea. Death occurred about 12 hours after the single dose of 0.5 gm. Subsequent autopsy showed much of the nitrobenzene still in the crop. The entire gastrointestinal tract was covered with large inflamed areas, and the feces were speckled with blood.

The excreta were dried and extracted with absolute alcohol, the alcoholic solution was evaporated by means of an electric fan, and the residue taken up with water. An emulsion remained, and only qualitative tests could be made, but by means of the indophenol reaction and the oxyazo color reaction, the presence of *p*-aminophenol was assured. For the former test, a small amount of the above mentioned water solution was taken, and strongly acidified with hydrochloric acid. Then a few drops of freshly prepared calcium chloride solution were added. A red tinge appeared, which turned to blue when the contents of the test-tube were made strongly alkaline with ammonia. This test indicates the presence of *p*-aminophenol. In order to confirm this test, a few drops of the same water solution were taken, made strongly acid with hydrochloric acid, and cooled in ice water. Then a few drops of a 1 per cent sodium nitrite solution were added, together with a drop or two of an alcoholic solution of α-naphthol. Finally, the entire solution was made strongly alkaline with ammonia, whereupon a red color appeared. As this also denotes the presence of *p*-aminophenol, it is safe to say that at least small amounts of this substance were formed.

o-Nitrophenylpropiolic Acid.

A large rooster received 0.5 gm. of this acid once a day for 6 days. Later 10 gm. more were fed to three birds during a period of 4 days, so that each bird received between 0.5 and 1 gm. of the acid per day. No ill effects were noted in any of the cases.

As to chemical behavior, we had rather expected that a reduction would take place both in the side chain as well as in the nitro group of the ring, and that a molecule of carbon dioxide would be split out, resulting finally in the production of indigo. Such was the reaction, it will be recalled, which was observed by Hoppe-Seyler (10) after he had fed this compound to rabbits. The excreta, therefore, were carefully dried, extracted with absolute alcohol in order to take up as little of the inorganic matter as possible, and then allowed to evaporate spontaneously. As no blue color was observed any time during these operations, it was thought that perhaps the compound had been excreted in the form of indoxyl joined with some other radical, such as glycuronic acid or $-KSO_4$, as is the case with indican in the human body. Accordingly, some of the residue from the alcoholic solution was taken up in water and treated with an equal volume of concentrated hydrochloric acid containing a small amount of ferric chloride. This is known to oxidize indican to indigo, and is the test for the determining, both qualitatively and quantitatively, as indigo the indican in human urine. Neither in this case, however, nor in other cases where other oxidizing agents were used, was there any sign of indigo formation. Various tests were then made to determine the presence of an unsaturated compound, but this also brought negative results. Hence it was concluded that little if any of the *o*-nitrophenylpropiolic acid had passed through the organism unchanged.

The residue from the alcoholic extract was then acidified to Congo red with phosphoric acid, and extracted repeatedly with ether in a rotary extraction apparatus. The ether extract was allowed to evaporate spontaneously. No crystals, however, appeared during the evaporation, and only a yellow oil remained at the end. This yellow oil was then taken up in hot water, boiled with animal charcoal, and filtered hot. On cooling, there appeared a group of leafy, white crystals which settled to the bottom, not unlike benzoic acid crystals. The crystals were filtered, recrystallized from hot water, dried at 60°C. *in vacuo,* and found to melt at 146–148°C. Analysis showed that the compound was *o*-nitrobenzoic acid.

Analysis.

0.1414 gm. substance: 0.2607 gm. CO_2 and 0.0420 gm. H_2O.
0.1263 " " 9.30 cc. N at 19°C. and 756 mm. pressure.

Calculated.	C 50.30,	H 3.00,	N 8.38.
Found.	" 50.27,	" 3.19,	" 8.39.

m-Aminobenzoic Acid.

We fed a chicken 1.5 gm. of this acid, as a solution of the sodium salt, daily for 3 consecutive days. As in the former experiments, large amounts

of hemp-seed were placed at the disposal of the fowl, to furnish ornithine for conjugation, should it be required. The combined excreta were extracted with hot 95 per cent alcohol, as in the case of the *p*-hydroxybenzaldehyde. The fat-free filtrate was acidified to Congo red with hydrochloric acid, whereupon there appeared immediately a flocculent mass of brownish yellow needles. These crystals were filtered off, recrystallized from hot water, dried at 80°C. *in vacuo*, and were then found to melt at 247–249°C. As this melting point seemed to indicate *m*-acetylaminobenzoic acid, we tested the material in the following manner.

Some of the compound was heated with a few drops of concentrated sulfuric acid together with a little ethyl alcohol. Thereupon, the odor of ethyl acetate was plainly observable. Another sample of the substance was boiled with a mineral acid under a reflux condenser for some time. The acetyl radical was thus split off, and the *m*-aminobenzoic acid thus formed was isolated, recrystallized from hot water, dried, and found to melt at the proper point; namely, 172–174°C. We obtained 1.8 gm. of the *m*-acetylaminobenzoic acid from the excreta after feeding 4.5 gm. of *m*-aminobenzoic acid.

DISCUSSION.

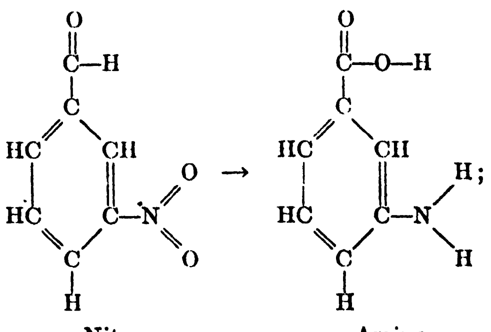
The fowl has a chemical protective mechanism which acts, in many ways, differently from that of the other animals as well as from that of man. Still it has in common with both of these the power to oxidize aldehydes to acids. The detoxication of benzaldehyde is accomplished by exactly the same reaction as that found in the cases of man, dogs, and rabbits, inasmuch as the benzaldehyde is first oxidized to benzoic acid. The detoxication of the benzoic acid, however, is then effected in the hen through the formation of ornithuric acid, namely by combining 2 molecules of benzoic acid with 1 molecule of ornithine (α-δ-diaminovaleric acid), while in man, as well as in the brute animals named above, the benzoic acid is joined with glycocoll and excreted in the urine as hippuric acid.

p-Hydroxybenzaldehyde, in the organism of the fowl, is likewise oxidized to the corresponding acid, but the acid thus formed is not joined with ornithine, for it is apparently very slightly toxic. Inasmuch, therefore, as the *p*-hydroxybenzoic acid is not conjugated, its fate in this case is comparable to the treatment it receives in the organisms of the dog, rabbit, and monkey (11). In the human body, however, the greater part of the *p*-hydroxybenzoic acid is combined with glycocoll, forming *p*-hydroxyhippuric acid (12).

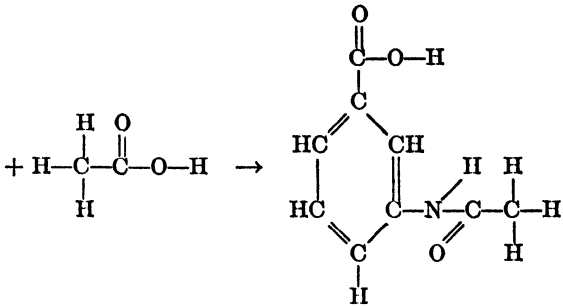
Phenylpropionic acid, when fed to chickens, suffers the same initial fate as when it is fed to other animals, *i.e.* it undergoes β-oxidation, by which it is converted into benzoic acid. Dakin (13) has pictured this reaction as taking place through several intermediate steps; namely, phenyl-β-hydroxypropionic acid, benzoylacetic acid, acetophenone, and finally benzoic acid. All of these steps, with the possible exception of the acetophenone, are well established facts, and no doubt the identical reactions take place in the organism of the fowl.

Cinnamic acid, likewise, undergoes much the same reaction when fed to fowls as when fed to other animals, for there is ultimate oxidation to benzoic acid. The benzoic acid is then combined with ornithine and excreted as ornithuric acid. Still, we were unable to isolate any of the intermediary products, though the reaction *in toto* is probably the same as that taking place when cinnamic acid is fed to dogs and to cats. It is questionable, however, whether the acid is first converted by reduction into phenylpropionic acid and then oxidized, or whether the first compound to be formed is either phenyl-β-hydroxypropionic acid or phenyl-β-ketopropionic acid.

m-Nitrobenzaldehyde shows rather a wide divergence in its catabolic pathway through the organisms of the dog and man from that followed in the case of the rabbit. When fed to rabbits (14), there is a simultaneous oxidation and reduction, by which the aldehyde is converted into a carboxyl group, and the nitro into an amino group, with a subsequent acetylation of the *m*-aminobenzoic acid. The reaction is represented in the following manner.



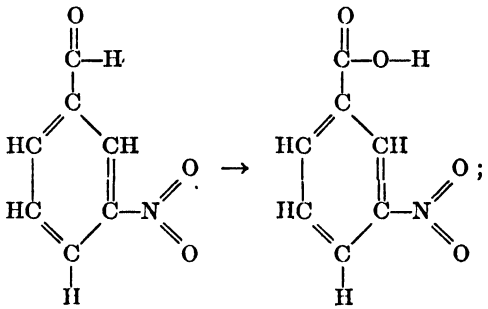
m-Nitrobenzaldehyde. *m*-Aminobenzoic acid.



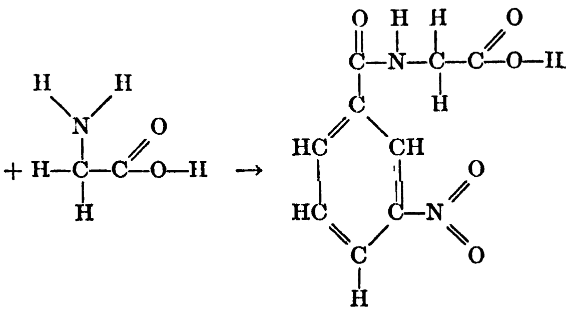
Acetic acid. *m*-Acetylamino-benzoic acid.

When *m*-nitrobenzaldehyde, however, is fed to dogs (15), there occurs merely an oxidation of the aldehyde group to a carboxyl group. The *m*-nitrobenzoic acid thus formed, is then joined with glycocoll, and appears in the urine as *m*-nitrohippuric acid.

Man detoxicates this compound in the same manner as does the dog (16), namely, as follows:



m-Nitro-benzaldehyde. *m*-Nitro-benzoic acid.



Glycocoll. *m*-Nitro-hippuric acid.

The chicken, however, apparently detoxicates this substance by forming a compound different from the end-product found either in the case of the dog or of the rabbit. Nor is it an ornithine conjugate, but appears rather to be an inner anhydride of *m*-aminobenzoic acid.

Nitrobenzene is apparently detoxicated by the fowl in exactly the same way as by other animals and by man. It undergoes a reduction of the nitro group to an amino group, with a simultaneous oxidation in the *p* position, forming a phenol. The resulting *p*-aminophenol is much less toxic than the nitrobenzene, but unfortunately it is impossible to feed large enough amounts of the nitrobenzene to be able to study the question satisfactorily, for when large doses are fed, the subject lives only a few hours, and consequently most of the material remains in the gastrointestinal tract.

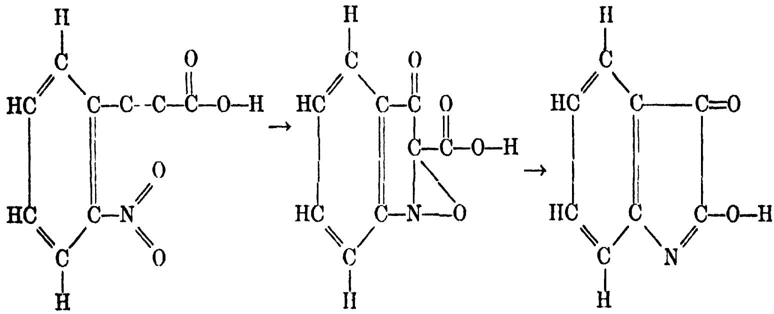
o-Nitrophenylpropiolic acid furnishes another example where there is the possibility of a reduction of a nitro group. Furthermore, the acid contains an unsaturated side chain, thus providing an additional opportunity for reduction. Hoppe-Seyler, who was the first to work with this compound, found, that although it was somewhat toxic to rabbits, still he was able to feed 1 to 3 gm. per day to a large rabbit until a total of 27 gm. had been fed without killing the animal (10). With dogs, however, the case was quite different, for when the same dose was administered to even fairly large animals, they invariably died in a very short time. They developed a marked case of polyuria and of temporary glycosuria after each dose. The rabbits, however, exhibited none of these symptoms. In the case of rabbits and probably also in that of dogs, there is a reduction of the nitro group to an amino group, with a simultaneous reduction in the side chain. The side chain is thus converted from a triple bonded compound to a double bonded compound (propiolic acid to acrylic acid). The next step is probably the closing of the ring to form, first indolecarboxylic acid, then indoxyl, and lastly indican. The reaction is illustrated below.

When *o*-nitrophenylpropiolic acid was fed to a chicken in 0.5 to 0.75 gm. doses, only slight signs of toxicity were exhibited. Moreover, there was no reduction of the nitro group, but apparently only a reduction of the triple bond of the side chain to a

double bond. The *o*-nitrophenylacrylic acid thus undoubtedly formed, was then oxidized, according to the scheme of β-oxidation, to *o*-nitrobenzoic acid and excreted uncombined.

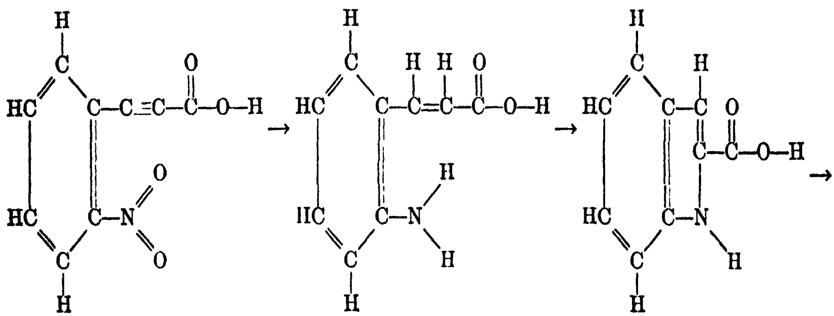
It is interesting here to compare the processes of reduction and subsequent oxidation of *o*-nitrophenylpropiolic acid which occur within the organisms of the fowl and the rabbit, with those which take place in the chemical laboratory.

Change Taking Place in Alkaline Solution in the Laboratory.

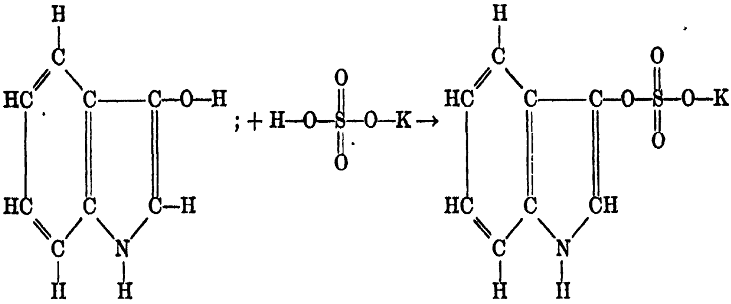


o-Nitrophenylpropiolic acid. Isatogenic acid. Isatin.

Change Occurring in the Organisms of the Dog and Rabbit.

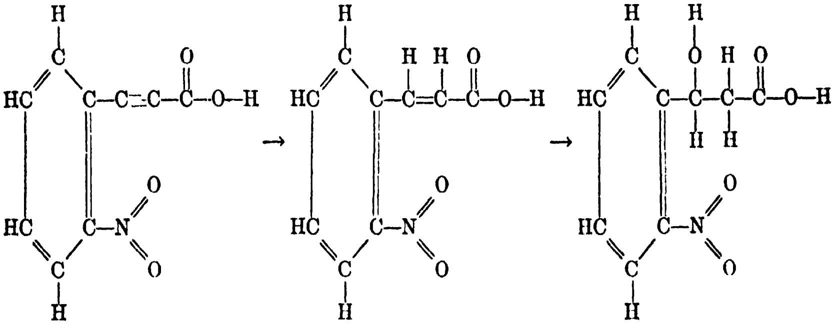


o-Nitrophenylpropiolic acid. *o*-Aminophenylacrylic acid. Indole-2-carboxylic acid.

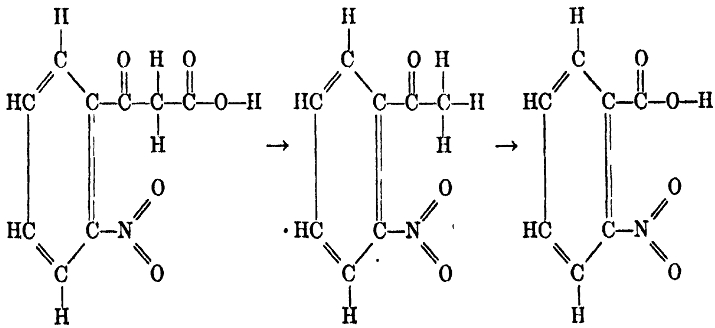


Indoxyl. Potassium hydrogen sulfate. (Indican) indoxyl potassium sulfate.

Change Occurring in the Organism of the Fowl.



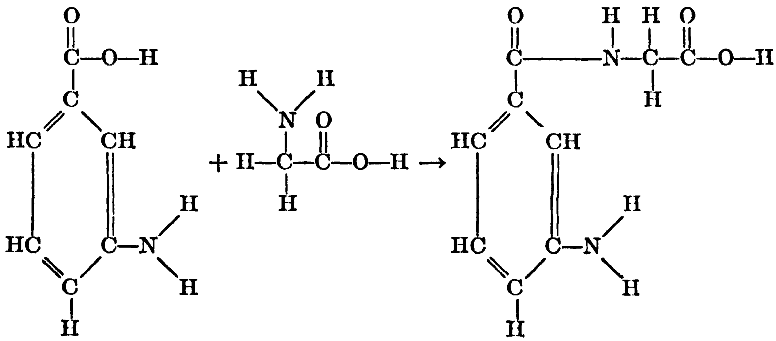
o-Nitrophenylpropiolic acid. *o*-Nitrophenylacrylic acid. *o*-Nitro-β-hydroxyphenylpropionic acid.



o-Nitrobenzoylacetic acid. *o*-Nitroacetophenone. *o*-Nitrobenzoic acid.

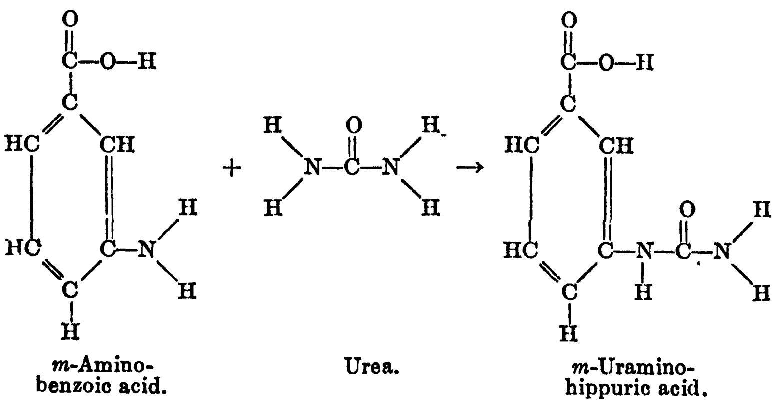
m-Aminobenzoic acid is perhaps the least toxic of all the compounds fed, and, therefore, it is excreted in the urine of the dog and the rabbit mostly in the form of the sodium salt. Salkowski (17), however, reports that in the urine of the dog some of the acid appeared in the form of *m*-uraminobenzoic acid, and Hildebrandt (18) claims to have found some joined with glycocoll as *m*-amino-hippuric acid. As the chicken has no urea at its disposal to form uramino-acids, and no glycocoll to form hippuric acid, it was necessary for the bird either to excrete the *m*-aminobenzoic acid free, or to detoxicate it in some other manner. Acetylation was the means employed.

Fate of m-Aminobenzoic Acid in the Organism of the Dog.



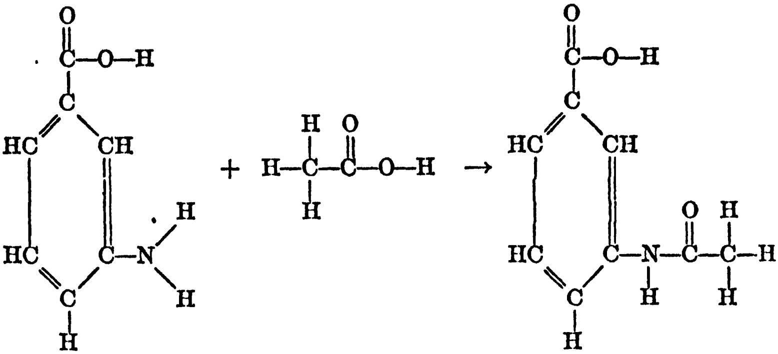
m-Amino-benzoic acid. Glycocoll. *m*-Amino-hippuric acid.

Also:



m-Amino-benzoic acid. Urea. *m*-Uramino-hippuric acid.

Fate of the Same m-Aminobenzoic Acid in the Body of the Fowl.



m-Amino-benzoic acid. Acetic acid. *m*-Acetylamino-benzoic acid.

The fowl has at its disposal, for the detoxication of groups of compounds, many of the ordinary reactions which are common to other animals. It is able to oxidize aromatic acids according to the rule of β-oxidation, and to reduce aromatic nitro compounds, with or without simultaneous oxidation within the same molecule. It is able to methylate and to acetylate. Apparently it cannot form uramino compounds, nor furnish glycocoll for conjugation purposes. However, it can furnish ornithine in relatively large quantities—a reaction which, as far as we know, is the exclusive property of the bird. We are investigating at the present time the question concerning the source of ornithine in the avian body. Our findings so far seem to indicate that it can be synthesized on demand for detoxication work—results which correspond with the facts we have already shown to be true regarding glycocoll and glutamine in the human body (19). Two other detoxication reactions remain for investigation; namely, can the bird employ the sulfate radical as a means of detoxication, and can it make use of glycuronic acid for similar purposes?

SUMMARY.

Previous work has shown that the fowl has two methods of defence against foreign chemical compounds, (1) by methylation, and (2) by conjugation with ornithine. We fed the following substances, in order to test out general type reactions in the

organism of the fowl: benzaldehyde, *p*-hydroxybenzaldehyde, phenylpropionic acid, cinnamic acid, *m*-nitrobenzaldehyde, nitrobenzene, *o*-nitrophenylpropiolic acid, and *m*-aminobenzoic acid.

It was found that benzaldehyde was oxidized to benzoic acid, and then conjugated with ornithine to form α-δ-dibenzoyl ornithine (ornithuric acid). *p*-Hydroxybenzaldehyde was oxidized to *p*-hydroxybenzoic acid and excreted in this form. The human organism in a like manner excretes *p*-hydroxybenzoic acid uncombined, but in the lower animal organisms the substance is conjugated in part to form *p*-hydroxyhippuric acid.

Phenylpropionic acid first underwent β-oxidation, with the formation of benzoic acid, as occurs also in the cases of man and the lower animals, and was then conjugated and excreted as ornithuric acid. Cinnamic acid was also oxidized to benzoic acid and eventually excreted as ornithuric acid. *m*-Nitrobenzaldehyde is converted by the rabbit into *m*-acetylaminobenzoic acid, and by the dog, into *m*-nitrohippuric acid. In the fowl the compound is apparently reduced and oxidized simultaneously, but is not conjugated. The resulting compound was not isolated, but it is suggested that an inner anhydride of *m*-aminobenzoic acid was formed.

Nitrobenzene suffered the same fate in the organism of the chicken as in all other experimental animals thus far employed. It was converted into *m*-aminophenol. *o*-Nitrophenylpropiolic acid, when fed to dogs or rabbits, is converted into indican (indoxyl potassium sulfate), while in the fowl it merely underwent β-oxidation of the side chain, and was thus converted into *o*-nitrobenzoic acid. *m*-Aminobenzoic acid is converted by the dog into either *m*-uraminobenzoic acid, or *m*-aminohippuric acid, or it is excreted free, while in the fowl it is acetylated to form *m*-acetylaminobenzoic acid.

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A STUDY OF THE ADEQUACY OF CERTAIN SYNTHETIC DIETS FOR THE NUTRITION OF PIGEONS.

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In spite of the fact that since the early work of Eijkman (1) pigeons have been widely used for a study of the antineuritic vitamine, no study of the general nutritive requirements of this species has been reported. Indeed, with exception of the antineuritic vitamine, very little is known of the rôle of the food accessories in avian nutrition.

Funk (2) has reported that beri-beri may be developed in pigeons by feeding a synthetic diet. This investigator employed a modification of the Osborne and Mendel (3) diet which consisted of casein, fat, starch, sugar, and salts. Addition of a yeast extract to the diet prevented the onset of beri-beri.

Drummond (4) was unable to rear young chicks in the laboratory. Osborne and Mendel (5), however, have successfully reared chickens in laboratory confinement. These investigators suggest that the difficulties usually encountered here are due to insufficient consumption of roughage and lack of exercise.

Very recently Emmett and Peacock (6) have shown that chicks can be used satisfactorily in the study of vitamines A and B. They pointed out that the presence of vitamine A in their ration is imperative since absence of it terminates the lives of chicks in 14 to 21 days. Similar effects were noted by Hart, Halpin, and Steenbock (7). They showed that chicks required a liberal supply of the fat-soluble vitamine or more accurately those vitamines contained in cod liver oil.

Our present study is concerned with (*a*) the adequacy of a complete synthetic diet for the growth, maintenance, and reproduction of pigeons, and (*b*) with the question of the significance

of the fat-soluble vitamine in the nutrition of pigeons. The basic synthetic diet used consisted of casein 22, cane-sugar 10, starch 27, agar-agar 2, salt mixture 3 (8), butter fat 30, and yeast 6 per cent. This diet is designated as Ration A. It has previously been shown to be adequate for the normal growth of white rats (9).

The nature of the synthetic diet is such that pigeons are unable to pick up the foodstuff readily, therefore we adopted the method of forcible feeding. Ration A was easily rolled into small balls about the size of soy beans and these were hardened by placing them in a beaker immersed in cracked ice for 2 or 3 minutes. These balls of food were easily swallowed by the pigeons when placed one after another in the mouth.

Our pigeons were not especially selected for breed, being the mixed varieties commonly seen on the streets and roofs. Our normal diet consisted of a mixture of corn, Kaffir-corn, wheat, buckwheat, rice, barley, and hemp-seeds.

The pigeons, generally a pair, were kept in metal cages 20 × 24 × 12 inches. The top and the sides were made of wire netting. The floor was covered with sawdust. Some wooden excelsior was provided in each cage for nest-building. Fresh tap water and a mixture of broken charcoal and granite were given *ad libitum*. The room in which these cages were kept was well ventilated and lighted and a moderate temperature maintained.

The weight of each bird was recorded once or twice a week and its general behavior carefully noted. Body temperature was taken occasionally by placing a microthermometer into the pigeon's crop for 3 minutes. The average temperature of those pigeons fed upon synthetic diet Ration A was 107.5°F., and that for those fed upon mixed grain 107.9°F.

EXPERIMENTAL RESULTS.

Adequacy of a Synthetic Diet and the Amount of Food Required by Pigeons.—Experiments were made to determine the suitableness of Ration A for pigeons and at the same time to find the minimum quantity of the diet required.

Healthy adult pigeons were given a diet of polished rice *ad libitum*, and after about 2 weeks upon this diet some of the pigeons were changed to our normal food; while others were fed forcibly with daily rations of 5, 10, 15, and 20 gm. of Ration A. It was

found that the average daily gain in body weight in those fed upon 15 and 20 gm. of Ration A was equal to controls fed upon normal food; whereas consumption of 10 gm. of the food daily was slightly insufficient for normal gain in weight.

Another lot of healthy adult pigeons, but which were gaining weight slowly, were fed with daily rations of 15 gm. of Ration A. Control birds were allowed to eat normal food. Again it was found that 15 gm. of food were enough to produce normal growth in the pigeons.

Effect of Ration A upon Egg Production.—Pigeons 71 and 74, both females, were kept in the same cage for 34 days upon normal diet, and during that time the former pigeon laid 4 eggs, while the latter laid 2. The average weight of these 6 eggs was 19.8 gm. The yolks were of a normal golden yellow color.

The birds were then taken off the normal diet and were forcibly fed with a daily ration of 15 gm. of Ration A from April 5 until August 30, 1920, a total of 147 days. The results obtained from this experiment are given in Chart 1, from which it is clearly seen that the pigeons grew well on the purified food after losing about 40 gm. in the first 10 days. It may be noted that in later experiments no other pigeons lost body weight at the beginning of a similar diet, and these birds were, therefore, exceptions in this respect. It is interesting to note that these pigeons after having ceased egg production during 50 days immediately following the change in their diet began again to lay eggs. Since the birds were both females and would not have fertile eggs, we removed the eggs from the cage as soon as they were discovered, and weighed them. During the 147 days on the synthetic diet Pigeons 71 and 74 produced 18 eggs, having an average weight of 19.5 gm. each. This egg production is normal. No abnormalities were found in eggs from the birds on the synthetic diet except that the color of the egg yolk in these cases was pale yellow instead of the normal golden yellow color when the same pigeons were fed upon our mixed grain diet.

As regards the cause of the pigmentation in the egg yolk of birds the following observations may be cited. Palmer and Kempster (10) have observed that the pigmentation of the egg yolk from White Leghorn hens raised upon a carotinoid-free diet was very much reduced, but the yolks were not absolutely

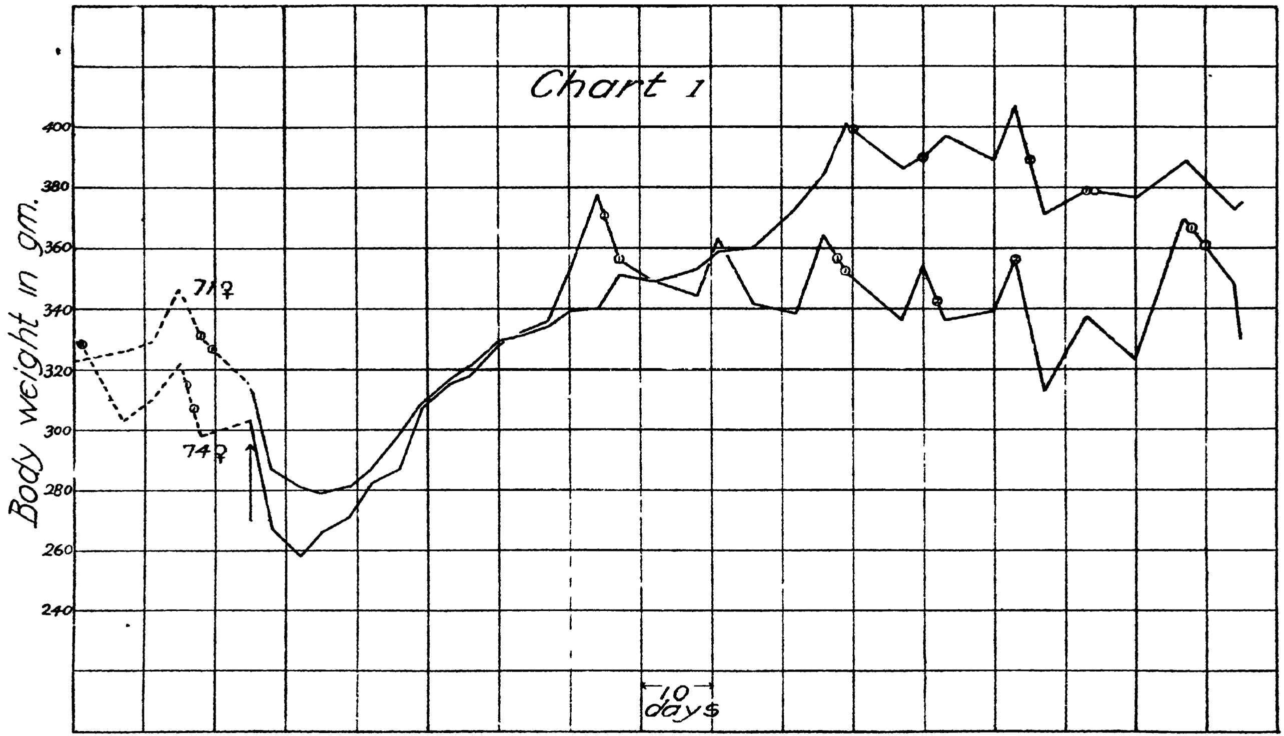

Chart 1. The curves show that two adult female pigeons maintained body weight and laid eggs freely on a synthetic diet consisting of casein 22, cane-sugar 10, starch 27, agar-agar 2, salt mixture 3, butter fat 30, and yeast 6 per cent. At the point marked by the arrow (↑) normal food was changed to the above diet. The circles indicate the time of laying eggs.

colorless. The residual pigment in the yolks was, therefore, not derived from plant carotinoids. Steenbock (11) and Steenbock and Boutwell (12) have called attention to the fact that yellow maize, carrots, and yellow sweet potato contain relatively much larger amounts of fat-soluble vitamine than do the white maize, mangel, sugar beet, red beet, parsnip, dasheen, rutabaga, and potato, and suggest that the fat-soluble vitamine is associated with the yellow pigments. The presence of a very large amount of fat-soluble vitamine in chicken's egg yolk has been reported by McCollum and Davis (13) and Osborne and Mendel (14). Palmer (15, 16) mentions a few instances where Steenbock's inference that the fat-soluble vitamine is a yellow plant pigment or a closely related compound does not hold. For he was able to raise chickens from hatching to maturity on a diet almost free from carotinoids; and mentions that substances with high fat-soluble vitamine content, namely milk fat of sheep, swine, dogs, cats, rats, rabbits, and guinea pigs is colorless; and that while the highly colored (golden yellow) cottonseed oil is rich in carotinoids, it is lacking in the vitamine factor.

Adequacy of the Synthetic Diet for Reproduction and Growth.—In the present experiment we mated female pigeons with male pigeons to determine whether the eggs were capable of fertilization and whether the young produced would grow upon the synthetic diet.

Pigeons 70 and 73, male and female, while on the normal diet, produced two sets of squabs over a period of 80 days. The growth curves of the first nestlings are shown on Chart 2. Pigeons 70 and 73 were then placed on our synthetic diet, Ration A. 15 gm. of this diet were fed daily. 4 days after the removal of the second pair of squabs the female laid the third pair of eggs. These eggs were left in the nest to determine the effect of the disturbance of incubation by the daily handling of the pigeons. The pigeons hatched the eggs but took about 13 days longer than the normal incubation time. The nestlings were removed from the adults. 12 and 14 days later the female laid an egg. These weighed 18.65 and 17.70 gm., respectively. Both eggs were cared for by the adults and were hatched 16 and 18 days later. This incubation time is normal.

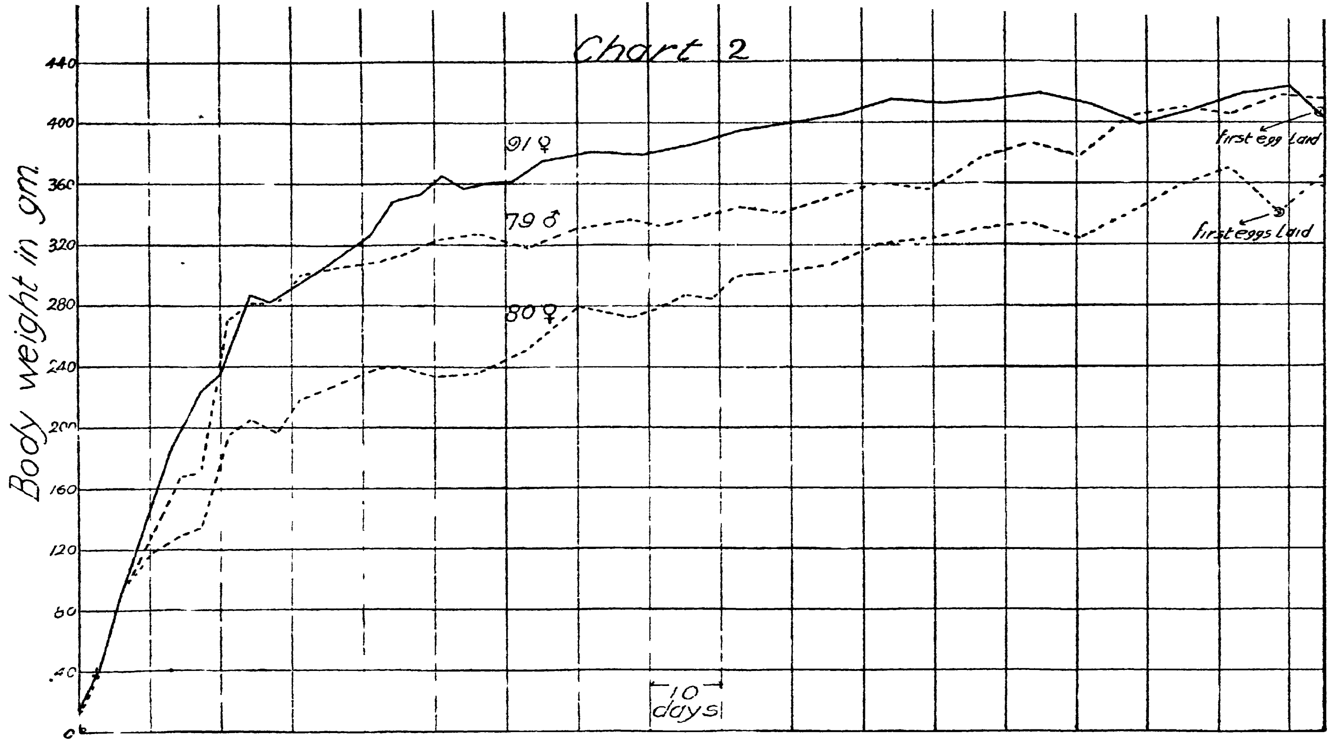

CHART 2. Comparison of growth in body weight of pigeons from birth to maturity on normal food with that of a bird fed artificially on a synthetic diet which consisted of casein 22, cane-sugar 10, starch 27, agar-agar 2, salt mixture 3, butter fat 30, and yeast 6 per cent.

The broken lines indicate those upon normal diet.

From this time until the separation from the young 30 days later the parents were given 20 gm. of Ration A daily. One nestling died the day after hatching; while the other of the pair appeared in excellent health. Its growth was normal throughout, as illustrated in Chart 2. Other experiments have yielded similar results. No leg or wing weakness could be detected at any stage in these birds. Careful observation upon pigeons raised upon the synthetic diet fails to show any definite abnormality in regard to general appearance, shade, and luster of feathers, except that the visible skin of the legs has an anemic appearance and fails to show the natural red pigmentation. Osborne and Mendel (5) have pointed out that lack of xanthophyll or carotin in diets causes the absence of pigmentation in the epidermal scales on the legs and ear lobes of chickens.

The Importance of Fat and Fat-Soluble Vitamine in the Nutrition of Pigeons.—Ration B was made up of casein 22, cane-sugar 10, starch 37, agar-agar 2, salt mixture 3, yeast 6, and lard 20 per cent. Ration C contained casein 22, cane-sugar 10, starch 27, agar-agar 2, salt mixture 3, and yeast 6 parts. A certain amount of cane-sugar solution (100 cc. of water added to 120 gm. of cane-sugar) was added to Ration C (for every 12 gm. of Ration C, 4 cc. of the sugar solution were added).

Experiment 1.—Four pigeons, two freely laying females and two males, were forcibly fed with a daily ration of 12 gm. of fat-free diet, Ration C, for 60 days and then their diet was changed to a daily ration of 15 gm. of the complete synthetic diet, Ration A. It was found that during the period of the first 60 days on a fat-free diet, Ration C, the pigeons maintained their body weights, appeared in good health, showed neither ruffled feathers nor any sign of an eye infection, yet the females failed to lay eggs. Ration C, as here employed, is inadequate, since when the diet was changed to Ration A, which contained both fat and fat-soluble vitamine the pigeons not only promptly gained weight but they also resumed egg production. The points involved were studied further as follows:

Experiment 2.—When two adult pigeons, male and female, appeared with the characteristic symptoms of polyneuritis caused by the natural feeding on polished rice they were forcibly fed with 12 gm. of Ration C daily. The birds increased in weight, but subnormally. During the period of 48 days the female bird did not lay any eggs. However, when the pigeons were allowed to eat normal food, the female laid eggs very frequently.

Experiment 3.—Two adult pigeons, male and female, were artificially fed with a daily ration of 15 gm. of a diet free from water-soluble B vitamine, which contained casein 22, cane-sugar 10, starch 33, agar-agar 2, salt mixture 3, and butter fat 30 per cent. The birds developed marked symptoms

of polyneuritis on the 22nd day. At this point their diet was changed to daily forcible feeding of 12 gm. of Ration C. The result showed that this latter diet not only prevented the birds from dying; but also gave to them a nearly normal increase in weight. However, the female bird failed to produce eggs during a period of 29 days. When her ration was changed to a daily amount of 15 gm. of Ration B which contained 20 per cent of lard she soon laid eggs.

Experiment 4.—The following experiment gives further proof of the adequacy of Ration B in which fat is supplied by lard. Here the normal diet of the adult pigeons, male and female, was changed to Ration B 1 day after they hatched eggs. At this time the parents were artificially fed with a daily ration of 20 gm. of this mixture and kept on it for 9 days, then the amount of daily intake was increased to 30 gm. and they were kept on it for 11 days. At this time 10 gm. of the mother's ration were given to the growing squabs. These daily rations were maintained for 5 days longer and then 20 gm. were given to each adult pigeon and 15 gm. to the squabs. At the end of 30 days from the time the eggs hatched, the squabs were separated from their parents and fed with a daily ration of 15 gm., the same amount as the adults. In these squabs, Nos. 107 and 108, there were observed no marked abnormalities. Their body growth was somewhat slow and the growth of feathers was somewhat delayed.

Above we have shown that a daily ration of 15 gm. of Ration A, which contains casein 22, cane-sugar 10, starch 27, agar-agar 2, salt mixture 3, butter fat 30, and yeast 6 per cent was enough to give to pigeons their normal growth and to allow them to produce fertile eggs. This amount of food, if completely burned, would give about 77 calories.

In the preceding experiments we have also shown that a daily ration of 15 gm. of Ration B which contains 20 per cent lard instead of 30 per cent butter fat in a synthetic diet gives very satisfactory results for the growth and egg production of pigeons. This daily amount furnishes about 69 calories. However, when the birds were artificially fed with a daily ration of 12 gm. of fat-free diet, Ration C, together with 4 cc. (contained 2.74 gm. sugar) of cane-sugar solution the growth of the pigeons was below normal and the females failed to lay eggs. This quantity of food when oxidized in the body would supply about 53 calories.

In the present experiment we studied whether by increasing the amount of the daily ration and in this way the caloric value the fat-free diet, Ration C, could be made adequate.

Experiment 5.—Two adult pigeons, male and female, whose diet had been Ration B, were changed to a fat-free diet, Ration C, and they were fed artificially 18 gm. of it and 8 cc. of our cane-sugar solution daily. The

result of this experiment is interesting, since, contrary to the earlier results with 12 gm. of this diet, both pigeons grew normally and the female laid normal eggs regularly. These parents reared young successfully on a daily ration of 22 gm. of Ration C and 7.3 cc. of cane-sugar solution for each adult pigeon during the first 15 days. At this time their food was increased to 27 gm. and 9 cc., respectively, and thus continued for 15 days, when it was again reduced to the original amount, and at the same time Squab

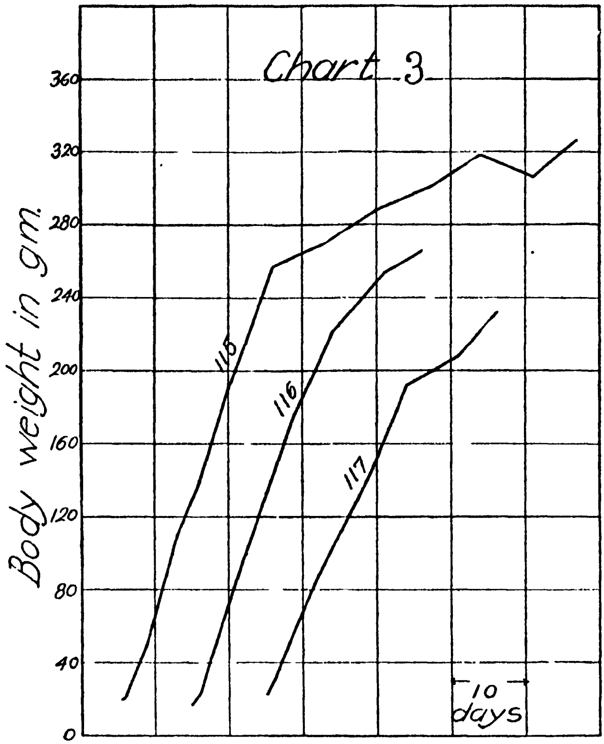

CHART 3. Curves showing the good growth of nestlings fed upon Ration C, which is free from fat and fat-soluble vitamine.

115 was removed from its parents and forcibly fed with a similar diet. The growth curve of the squab is given in Chart 3. Plate 1 shows photographs of Squab 115 at the age of 3, 10, and 29 days. Absence of any eye infection or other abnormality is evident.

Further similar experiments on the adequacy of Ration C, which is free from fat and fat-soluble vitamine, for rearing nestlings were carried out. It was found that the preceding findings were corroborated.

The experiments reported in the present paper present, as far as we know, the first instances recorded where normal growth, reproduction, and normal rearing of the young have been obtained upon highly purified diets plus a small amount of vitamine-containing material. The success with growth and reproduction in the absence of fat and fat-soluble vitamine will naturally raise the question of the significance of this latter food accessory in nutrition. It was only through taking into account the calories fed, and giving sufficient food by forcible feeding that we were able to eliminate fat and fat-soluble vitamine from the diet. We are now applying the same general line of study to this and related problems in white rats.

Our complete success with yeast as a sole source of vitamine B is perhaps of interest in connection with the recently reported work of Kennedy and Palmer (17) who state: "Our results do not support the general belief that yeast is an unusually valuable source of the growth-promoting vitamine B, or that it can be accepted as a standard product in experiments in which a vitamine B preparation is required." Kennedy and Palmer appear to us to have confused two essentially different questions in their discussion. These questions concern (*a*) the normal growth of white rats over considerable periods on a limited diet and (*b*) the question of continued maintenance and normal reproduction and rearing of young on a limited diet. In spite of the findings of Kennedy and Palmer we believe that the first of these questions is not open to dispute. Funk and Macallum (18), Benedict and Rahe (19), and very recently Osborne and Mendel (20) have reported normal growth of rats for long periods on limited diets with yeast as the source of vitamine B, and this experience is, we believe, common in most nutrition laboratories.

Concerning the second question, it may be noted that no one has reported normal reproduction and rearing of young in white rats upon the so called synthetic diets with simple addition of the recognized food accessories from restricted sources. The failure of Kennedy and Palmer in this connection is therefore not unexpected, and should not be interpreted as a basis for an indictment of yeast as a source of vitamine B. Such findings indicate that hitherto unrecognized food factors are necessary for the complete well being of animals. Our results show that this is not true for pigeons, and further work may cause a revision of our ideas on the

essential nature of some of the recognized food accessories in mammals. Kennedy and Palmer have failed to show that any other source of vitamine B is superior to yeast or even equal to yeast. Their few reported experiments with extract of wheat germ were not compared with results with an extract of yeast, and

TABLE I.

Effect on Eggs Following the Change of Diet of Parents from the Normal to One of 18 Gm. of Fat-free Diet, Ration C.

Experiment No.	Pigeon No and sex.	Duration of artificial feeding.	No of fertile eggs.	No of sterile eggs.	Remarks.
		1921			
1	103♂ 73♀	Jan. 28 to Apr. 14	8	0	The third pair of eggs was allowed to hatch and rear. 21 days after the third pair hatched the female laid a fourth pair.
2	110♂ 111♀	Mar. 1 to Apr. 14	3	1	
3	101♂ 91♀	Mar. 4 to Apr. 14	4	0	The second pair of eggs was allowed to hatch and the young were reared.

the growth they obtained with the wheat extract is apparently not superior to that commonly obtained with yeast as a source of vitamine B.

SUMMARY.

1. A synthetic diet consisting of casein 22, cane-sugar 10, starch 27, agar-agar 2, salt mixture 3, butter fat 30, and yeast 6 per cent; or a synthetic diet containing casein 22, cane-sugar 10, starch 37, agar-agar 2, salt mixture 3, yeast 6, and lard 20 per cent is an adequate diet for fertile egg production, growth, and maintenance of common pigeons.

2. Daily rations of from 15 to 20 gm. of these synthetic diets are sufficient to promote normal growth in squabs.

3. If a diet contains proper proportions but supplies too few calories, pigeons upon that diet may not maintain their normal weight and do not lay eggs.

4. Pigeons on a diet of sufficient caloric value, even though the diet lacks fat and fat-soluble vitamine, may maintain excellent condition, and may produce fertile eggs and rear healthy squabs. *Hence fat-soluble vitamine is not essential in any stage of avian nutrition.*

5. An adequate food for pigeons should contain about 70 calories in the daily ration.

6. From the fact that the diets employed contain no antiscorbutic vitamine it is safe to say that the pigeon requires no antiscorbutic vitamine.

7. Common pigeons can be raised normally under strictly laboratory conditions and may, therefore, render service for the study of nutritional problems.

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EXPLANATION OF PLATE 1.

Showing three stages of a pigeon hatched and reared on a fat-free and fat-soluble vitamine-free diet.

POTASSIUM IN ANIMAL NUTRITION.

I. INFLUENCE OF POTASSIUM ON URINARY SODIUM AND CHLORINE EXCRETION.*

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Among the mineral elements which have been studied extensively in relation to their various physiological rôles is the metal potassium. Ringer (1) was the first to show the importance of potassium in maintaining the normal heart beat and later Howell (2) showed that the vagus nerve lost its power of inhibiting the beat of the heart if potassium was absent. Zwaardemaker (3) has advanced the idea that the indispensability of potassium in cardiac action is due to the slight radioactivity of the element. He has demonstrated that other radioactive substances as thorium, uranium, ionium, and radium can replace the potassium ion in restoring the heart beat after the heart has stopped beating in a Ringer solution containing no potassium.

R. F. Loeb (4) does not agree with Zwaardemaker that the physiological action of potassium in cardiac action is due to its radioactivity. On the basis of Lingle's (5) experiments who showed that an increased supply of oxygen can resuscitate a heart which has ceased to beat in a pure sodium chloride solution, Loeb (4) argues that oxygen is activated in some other form when the penetrating rays from uranium, thorium, and radium go through the heart and that this may explain the restoration of the heart beat without having to assume that potassium, rubidium, and cesium act directly physiologically by virtue of their radioactivity. J. Loeb (6) concludes from data obtained by him that

* Part of a thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at the University of Wisconsin.

the behavior of the potassium ion in antagonistic salt action is due to its purely chemical character; *i.e.*, its position in the periodic system or rather its atomic number and not to those explosions in its nucleus which give rise to a trace of radioactivity.

That the presence of potassium is necessary in the growth of microorganisms is an established fact and, furthermore, it is known that it cannot be replaced by the chemically related metal sodium. Herbst (7) and later R. F. Loeb (4) reported that both rubidium and cesium chlorides could replace potassium chloride in the development of sea urchins' eggs. As to what effect a ration low in potassium has upon the development of the mammal and also whether sodium can replace potassium in its physiological function are questions which are open to investigation. Investigations pertaining to the above are now in progress and will be reported in a later paper.

One of the most interesting and fundamentally important reactions of potassium in the animal body was pointed out by Bunge (8) in 1873. He attempted to attribute the craving which herbivorous animals have for common salt to an excess of potassium in the diet. This explanation is in harmony with many known facts. According to Bunge, when a salt, such as potassium citrate, gains entrance into the blood stream, a proportion of it reacts with sodium chloride, forming potassium chloride and sodium citrate. As the kidney functions in keeping the composition of the blood nearly constant, the abnormal constituents, sodium citrate and excess of potassium salts, are eliminated. In this manner, a high potassium intake may cause increased sodium and chlorine excretion.

Bunge demonstrated the effect of potassium salts by experiments upon himself which showed that the Na_2O increased 6.0 gm. and chlorine 3.4 gm. by adding 15.4 gm. of K_2O as potassium phosphate to his daily diet, but his experiments were not continued to ascertain what effect continuous addition of potassium salts would have upon sodium and chlorine excretion. He states, however, that undoubtedly a point would soon be reached at which the body would stoutly retain its remaining sodium. Bunge thought that the loss of the quantities of sodium and chlorine reported in his investigation appeared sufficiently large and that they should be replaced when a vegetarian diet containing an abundance of potassium is eaten.

As far as the writer was able to find to date, no one has conducted experiments to determine the effect of potassium on sodium excretion since 1873, the time of Bunge's carefully controlled experiment on himself. Although the high potassium intake occurred for only one 24 hour period, many text-books of physiology (9) and nutrition contain general statements and draw broad conclusions concerning the sodium and chlorine impoverishment brought about by a high potassium intake on the basis of this experiment.

In view of the limited data pertaining to sodium and chlorine excretion as influenced by a high potassium intake, it was decided to make a study of the effect of a diet containing a large amount of potassium upon sodium and chlorine excreted by way of the kidney. In order to have these results comparable to those obtained in practical feeding the above diet was continued for a period of several days. For this work the pig was used as the experimental animal because of its copious urinary excretion and its easy adaptation to changes in the diet necessary in this type of experiment.

EXPERIMENTAL.

The fact that a pig can maintain itself for several weeks on a starch diet gave the opportunity to study the effect of varying quantities of potassium under a condition of extreme mineral restriction with only the energy requirement being satisfied.

During the course of these experiments five different pigs were used and the diet was modified so as to obtain results under the same and varying conditions. The animals were fed twice a day, and the mixture of starch and water was prepared by pouring the starch into the boiling water and so regulating the quantity of water that a stiff jell resulted. Whenever a salt was added it was introduced in the water.

In all the experiments reported the urine was collected daily and aliquots were taken for potassium, sodium, and chlorine determinations. For potassium and sodium the urine was evaporated to dryness and heated to a dull redness in a porcelain dish. The residue was extracted with hot water, filtered, and the procedure followed was that given by Hawk (10). The sodium and potassium were weighed as chlorides and then used for determining

the potassium as potassium chloroplatinate. The chlorides were precipitated with 0.1 N silver nitrate in an aliquot portion of the

TABLE I.

Pig 1, male, weight 69 lbs. 500 gm. of starch fed daily.

Date.	Potassium given as potassium acetate.	Potassium excreted.	Sodium excreted.	Chlorine excreted.	Volume of urine.
1921	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*
Oct. 18		0.31	0.081		1,025
" 19		0.14	0.080		1,050
" 20		0.13	0.078		850
" 21		0.15	0.082		900
" 22		0.11	0.100	0.018	850
" 23	2.82	1.70	0.088	0.027	900
" 24	5.64	4.57	0.131	0.283	1,160
" 25		0.68	0.100		1,020
" 26		0.31	0.063		1,050
" 27		0.28	0.101		1,150
" 28		0.18	0.063		1,000
" 29		0.22	0.20	0.044	1,220
" 30	5.64	3.65	0.247	0.30	1,260
" 31	2.82	2.35	0.072	0.053	940
Nov. 1	5.64	4.68	0.146	0.177	1,140
" 2	5.64	4.96	0.136	0.088	1,050
" 3	5.64	4.87	0.118	0.088	785
" 4	5.64	5.18	0.325	0.30	945
" 5		1.03	0.162	0.038	1,100
" 6		0.47	0.112	0.018	1,100
" 7		0.22	0.075	0.018	1,070
" 8		0.37	0.119		1,050
" 9		0.19	0.102	0.018	930
" 10	11.28	6.80	2.540	2.080	1,500
" 11	14.10	11.28	0.820	0.600	1,050
" 12	14.10	12.60	0.250	0.320	950
" 13	14.10	12.81	0.137	0.150	1,070
" 14	14.10	12.72	0.200	0.370	1,150
" 15	14.10	13.92	0.530	0.580	1,150
" 16		2.54	0.156	0.060	670
" 17		0.64	0.134	0.060	780
" 18		0.38	0.237	0.018	1,130
" 19		0.27	0.282	0.018	935

urine, and the excess of $AgNO_3$ was titrated with ammonium sulfocyanate, using ferric alum as an indicator. In Table I are recorded the daily urinary excretions of potassium, sodium, and

chlorine for Pig 1. The potassium salts introduced through the periods of high potassium intake are also given.

On the starch diet the daily small quantities of potassium and sodium excreted do not exceed the quantities present in the starch consumed. When, however, extra potassium is given, 80 per cent or more of it appears in the urine within the period not exceeding

TABLE II.

Pig 2, male, weight 61 lbs. 500 gm. of starch fed daily.

Date.	Potassium given as potassium acetate.	Potassium excreted.	Sodium excreted.	Chlorine excreted.	Volume of urine.
	gm.	*gm.*	*gm.*	*gm.*	*cc.*
Oct. 26		0.110	0.312		1,030
" 27		0.120	0.630		1,300
" 28		0.055	0.560		1,270
" 29		0.077	0.370	0.476	1,220
" 30		0.087	0.114	0.159	1,050
" 31		0.047	0.100	0.018	1,140
Nov. 1		0.100	0.136	0.036	990
" 2		0.080	0.022	0.036	870
" 3		0.130	0.087	0.027	845
" 4	5.64	2.750	0.087	0.460	885
" 5		0.730	0.063	0.036	1,110
" 6		0.490	0.071	0.018	1,100
" 7					1,000
" 8		0.320	0.039	0.027	810
" 9		0.170	0.045	0.027	1,000
" 10	5.64	2.780	0.169	0.071	785
" 11	5.64	4.750	0.088	0.018	960
" 12	5.64	4.930	0.115	0.027	920
" 13	14.10	11.180	0.850	0.570	1,050
" 14	14.10	7.900*	0.068	0.064	515

*Starch-potassium acetate mixture not all consumed.

48 hours after the intake. Accompanying this high potassium in the urine there is an increase in sodium and chlorine, and while there is a remarkable increase in sodium and chlorine excretion during the first 24 hour period, the quantities daily excreted gradually fall off and in the case of sodium become equal to those daily excreted before extra potassium was introduced.

In Table II similar results are obtained due to potassium feeding under the same conditions, but employing another animal. In

addition there is a tendency for the quantity of chlorine excreted to become the same as the quantity eliminated by way of the kidney during low potassium intake.

For comparison, data obtained by Bunge (8) are presented in Table III. An immediate increase of sodium and chlorine in the urine due to the introduction of potassium phosphate in his food is seen. However, this is not permanent as in the following 3 days the sodium and chlorine excreted are relatively far lower than during the 3 days preceding the extra intake of potassium. As a result the average daily excretion of sodium oxide (as written at that time) for the 3 days preceding the test period was 2.209 gm., while from July 25 to 28, inclusive, it averaged practically the same; namely, 2.396 gm. For chlorine during the corresponding periods the respective amounts were 2.236 gm. as compared with 2.027 gm.

TABLE III.

Diet: 600 gm. of beef, 300 gm. of bread, 100 gm. of butter, 100 gm. of sugar, 2.0 gm. of salt, and 3 liters of water.

Date.	KO excreted.	NaO excreted.	Chlorine excreted.	Volume of urine.
	gm.	*gm.*	*gm.*	*cc.*
July 22	2.501	2.695	2.555	
" 23	2.472	2.088	2.224	2,366
" 24	2.580	1.846	1.930	2,294
" 25	13.290*	6.924	5.339	1,926
" 26	4.517	0.889	0.764	1,087
" 27	3.702	0.757	0.812	2,421
" 28	3.654	1.014	1.194	2,567

* 18.24 gm. of KO as K_2HPO_4 introduced in food.

As seen in this table the response recorded in Tables I and II is similar to that reported by Bunge. In addition, as the writer continued the administration of potassium salts, the results here reported show for the first time that even with such a continual administration of potassium the response of increased sodium excretion is only transitory. There is, however, more sodium and chlorine excreted during the potassium-starch period than during a corresponding period on starch alone.

In Tables IV and V additional data are given with two different pigs (Pigs 3 and 4) on the starch diet, giving the potassium first

as the phosphate and later as the acetate. The data in the first part of Table V where potassium phosphate was given are con-

TABLE IV.

Pig 3, male, weight 81 lbs. 600 gm. of starch fed daily.

Date.	Potassium added.	Potassium excreted.	Sodium excreted.	Chlorine excreted.	Volume of urine.
	gm.	*gm.*	*gm.*	*gm.*	*cc.*
Dec. 15		0.170	0.700		1,250
" 16		0.120	0.445	0.027	1,250
" 17		0.140	0.890	0.460	1,230
" 18		0.095	0.610	0.815	1,400
" 19	4.49*	0.130	0.180	0.220	1,030
" 20	6.73*	0.680	0.980	0.071	900
" 21	8.98*	2.150	0.250	0.195	1,150
" 22	8.98*	1.100	0.270	0.036	640
" 23		2 200	0.220	None.	1,480
" 24		0.270	0.810	0.018	1,310
" 25		0.160	0.114	None.	1,050
" 26		0.370	0.087	"	1,480
" 27	2.62†	0.700	0.114	0.142	1,325
" 28		0.640	0.110	0.248	1,400
" 29	11.28‡	7.950	3.080	4.110	2,100
" 30	11.28‡	11.600	0.580	0.910	1,900
" 31	11.28‡	11.710	2.110	0.740	1,870
Jan. 1	11.28‡	11.240	0.240	0.210	1,460
" 2	11.28‡	11.000	0.007	0.180	1,380
" 3§	11.28‡	10.650	None.	0.350	1,040
" 4	11.28‡	11.550	0.485	0.560	1,100
" 5	11.28‡	9.560	0.527	0.260	1,120
" 6	11.28‡	10.550	0.480	0.650	1,140
" 7	11.28‡	8.600	1.880	1.620	1,300
" 8	11.28‡	13.550	0.920	0.880	1,320
" 9	11.28‡	10.330	1.370	0.880	1,320
" 10					
" 11		1.46	1.430	0.072	1,700
" 12		0.46	0.230	0.027	1,860
" 13		0.23	1.250	0.018	1,420

* K_2HPO_4

† KCl

‡ $KC_2H_3O_2$

§ 2.0 gm. of NaCl given daily from Jan. 3 to 9, inclusive.

firmatory of those shown in Tables I and II where potassium acetate was used. Pig 3, Table IV, excreted only a small propor-

tion of the potassium, consumed as potassium phosphate, by way of the kidney, consequently the rate of sodium and chlorine excretion is not affected. This difference in potassium assimilation could not be explained as both pigs had been on the starch diet for about 2 weeks and appeared in good condition. The cathartic effect of potassium phosphate was more noticeable with Pig 3 than with Pig 4.

TABLE V.

Pig 4, male, weight 122 lbs. 600 gm. of starch fed daily.

Date.	Potassium added.	Potassium excreted.	Sodium excreted.	Chlorine excreted	Volume of urine.
	gm.	*gm.*	*gm.*	*gm.*	*cc.*
Dec. 27		0.16	0.230	0.008	1,450
" 28		0.17	0.140	0.032	1,400
" 29	6.73*	4.28	0.460	0.048	1,130
" 30	8.98	6.05	0.080	0.017	1,240
" 31	8.98	6.95	0.230	0.177	1,300
Jan. 1	8.98	7.02	0.420	0.089	930
" 2	8.98	6.90	0.360	0.064	1,260
" 3†	8.98	7.03	0.400	0.053	1,020
" 4	8.98	7.97	0.380	0.195	1,100
" 5	8.98	7.35	0.760	0.565	1,100
" 6	8.98	7.29	1.560	1.100	1,020
" 7	8.98	7.20	0.960	1.450	1,375
" 8	8.98	6.77	0.670	1.130	1,150
" 9	8.98	6.85	0.510	0.650	880
" 10					
" 11		1.47	0.490	0.570	1,900
" 12		0.76	0.400	0.320	1,400
" 13		0.62	0.280	0.160	1,540

* K_2HPO_4 from Dec. 29 to Jan. 10.

† 2 gm. of NaCl given daily from Jan. 3 to 9, inclusive.

As indicated in the tables the experimental procedure was modified in some cases during the high potassium intake by incorporating 2 gm. of sodium chloride into the diets. The purpose of this was to ascertain whether this additional sodium and chlorine would be promptly excreted. The data show there was no immediate increase of these elements in the urine and it was not until the 3rd day that the chlorine excreted was equal in quantity to the sodium chloride added to the ration.

In comparing the total quantities of sodium and chlorine excreted by the kidney during this period to the amounts taken in as sodium chloride by the two animals the following data are obtained; the intake of both animals was 5.51 gm. of sodium and 8.49 gm. of chlorine. Pig 3 excreted in a 7 day period 5.66 gm. of sodium and 5.21 gm. of chlorine. Pig 4 excreted 5.24 gm. of sodium and 5.14 gm. of chlorine. This shows practically a quantitative excretion of sodium, but only a 60 per cent excretion of the chlorine ingested. When potassium salts were previously introduced without sodium chloride, chlorine was eliminated in increased quantities promptly. Apparently, the potassium salts are not alone responsible when it comes to consider the chlorine balance, other factors must be given consideration.

In comparing the data of Pig 3 with those obtained on Pig 4 it furthermore appears that there exists no absolute quantitative relation between the amounts of sodium and potassium excreted. Both pigs excreted approximately the same amounts of sodium and also chlorine, yet Pig 3 excreted 10.68 gm. of potassium while Pig 4 excreted only 7.21 gm. of potassium. Though different salts of potassium had been fed to these pigs, additional data bearing out this particular point of lack of quantitative relations are brought out by inspection of Tables VI, VII, and IX.

Thus far the results reported have been with a starch diet plus additional potassium and sodium salts as indicated above. In order to obtain data under conditions where the diet was more nearly complete in satisfying the fundamental requirements for maintenance and growth the following synthetic ration was used.

	gm.	*Salt mixture.*	*gm.*
Casein	2,700	NaCl	50.0
Salt	579	$MgSO_4$	121.0
Yeast	300	Na_2HPO_4	26.0
Cod liver oil	150	$Ca_2H_2(PO_4)_2 \cdot 4H_2O$	280.0
Starch	13,200	Ca lactate	70.0
		Fe citrate	32.0

With such a diet we again find an increase in sodium and chlorine excretion during the 24 hour period following the intake of potassium acetate, and that these quantities of sodium and chlorine excreted daily become smaller in spite of the salt intake and although the potassium is kept at the same high level. The

average daily sodium and chlorine content of the urine is no greater during this 7 day period of high potassium intake in the case of each pig than it is during certain periods of low potassium intake.

The data of Pigs 3 and 4, Tables VI and VII, are presented in abbreviated form in Table VIII to help illustrate this point. The figures given for the period of high potassium from February 4 to 10 are taken for comparison as they are perhaps more representative of a certain period than any of the others. For example, Pig 3, Table VI, during the period from February 1 to 3 was probably storing chlorine to compensate for that excreted after the introduction of the potassium on January 26 and 27, which is similar to the process going on from February 6 to 10, although the high potassium feeding was being continued and the same quantity of potassium passed through the kidney. Comparing the quantities of sodium and chlorine in the urine to the quantities of the same elements in the salt mixture given, there appears to be a retention of these two elements during the latter part of the high potassium period as there was in Tables IV and V.

The ability of the animal to conserve its sodium and chlorine supply during the passage of large quantities of potassium through the blood is further shown in Table IX. This animal had received milk daily for 2 months and was in excellent condition. 5 liters of milk were given daily with additions of potassium acetate as indicated in the table.

The increase of potassium in the diet appears to bring about a slight increase in the sodium and chlorine content of the urine, but certainly no significant disturbance due to potassium feeding is effected. The average daily sodium excretions with increasing potassium intake for the different periods were as follows: December 15 to 17, 7.14 gm.; December 18 to 23, 4.74 gm.; December 24 to 29, 3.99 gm. In other words, as the potassium intake increases the sodium excretion through the kidney becomes less. Why this should occur is difficult to explain. It might be assumed that the mass action effect of this potassium acetate caused the formation of sodium acetate which in turn was eliminated by way of the intestine because no studies of the fecal excretion were made to exclude this possibility. The falling off in the sodium excretion must be due to a smaller degree of absorption by the blood stream. The animal, nevertheless, again shows its

protective property towards high quantities of potassium depleting the body of sodium as the nearly constant sodium chloride content

TABLE VI.

Pig 3. 627 gm. of ration fed daily.

Date.	Potassium added	Potassium excreted	Sodium excreted.	Chlorine excreted.	Volume of urine.
	gm	*gm*	*gm*	*gm.*	*cc.*
Jan. 14		0.190	0.102	0.027	900
" 15		0.180	0.490	0.088	1,480
" 16		0.190	0.760	0.212	980*
" 17		0.060	0.560	0.160	940*
" 18		0 080	0.190	0 160	1,200*
" 19		0.160	0.700	0.106	900*
" 20		0 120	0.610	0.355	1,300
" 21	9 55†				No urine excreted.
" 22		0.615	1.420	0.213	1,640
" 23		0.180	1.130	0.177	1,040
" 24		0.230	2.100	1.140	1,380
" 25		0.160	1.910	2.070	1,360
" 26	11.28‡	2.550	4.810	4.160	950
" 27	11.28	16.400	3 740	3.720	1,960
" 28	11.28	12.810	1.350	0.960	1,120
" 29		1.210	1.820	None.	1,000
" 30		0.200	0.320	"	1,060
" 31		0.150	0.280	"	1,140
Feb. 1		0.150	0.890	0.018	1,160
" 2		0 019	1.140	0.142	1,380
" 3		0 063	1.380	1.220	1,300
" 4	11.28‡	1.850	1.160	1.330	370
" 5	11.28	10.220	2.930	2.520	1,640
" 6	11.28	9.910	0.700	0.420	1,000
" 7	11.28	10.400	0.710	0.380	1,000
" 8	11.28	10.630	0.500	0.210	1,050
" 9	11.28	10.520	0.800	0.370	1,250
" 10	11.28	10.320	1.040	0.320	1,350
" 11		0.990	0.660	0.054	1,440
" 12		0.230	0.930	0.036	1,320
" 13		0.250	0.900	0.142	1,180
" 14				0.920	1,540

* Albumin in urine, hemoglobinuria.

† $C_6O_7H_5K_3$

‡ $C_2H_3O_2K$

of the blood gives the opportunity for the reaction with potassium salts to proceed.

The daily average chlorine excretions for the periods as mentioned above were: 4.83, 5.26, and 5.27 gm. Here, the tendency

TABLE VII.

Pig 4. 627 gm. of ration fed daily.

Date.	Potassium added.	Potassium excreted.	Sodium excreted.	Chlorine excreted.	Volume of urine.
	gm.	*gm.*	*gm.*	*gm.*	*cc.*
Jan. 14		0.47	0.73	0.230	1,000
" 15		0.34	0.49	0.620	1,100
" 16		0.26	1.30	1.170	1,100
" 17		0.19	0.48	1.080	950
" 18		0.18	1.05	1.030	1,200
" 19	9.56*	2.95	0.81	0.920	520
" 20		0.87	1.08	0.160	1,000
" 21		0.34	1.36	0.180	1,120
" 22	9.56*	4.87	1.52	2.030	980
" 23		0.55	1.98	0.430	1,150
" 24	9.56*	4.87	1.22	1.470	870
" 25	9.56*	6.79	0.63	0.900	1,050
" 26	11.28†	11.55	2.80	2.200	1,320
" 27	11.28†	11.61	2.13	1.290	1,160
" 28	11.28†	10.00	0.71	0.283	850
" 29		1.08	1.39	None.	1,170
" 30		0.41	0.90	"	
" 31		0.24	1.17	0.370	1,100
Feb. 1		0.20	1.42	1.010	1,250
" 2		0.21	2.02	1.860	1,300
" 3		0.17	1.31	1.490	1,350
" 4	11.28†	6.50	1.79	3.080	1,340
" 5	11.28†	9.66	1.46	1.340	1,200
" 6	11.28†	9.70	0.81	0.320	880
" 7	11.28†	10.00	0.36	0.120	1,060
" 8	11.28†	10.42	0.59	0.140	820
" 9	11.28†	10.80	0.71	0.120	1,300
" 10	11.28†	10.42	1.46	0.230	1,020
" 11		1.73	0.91	0.130	1,160
" 12		1.36	0.40	0.040	1,350
" 13		0.43	1.05	0.320	1,240
" 14				1.010	1,300

* $C_6O_7H_5K_3$

† $C_2H_3O_2K$

appears to be for chlorine excretion through the kidney to increase due to the increase of potassium excreted through the same organ.

TABLE VIII.

Pig 4. Period.	Average daily sodium excretion.	Average daily chlorine excretion.	Pig 3. Period.	Average daily sodium excretion.	Average daily chlorine excretion.
	gm.	gm.		gm.	gm.
Feb. 4–10 High potassium.	1.02	0.77	Feb. 4–10 High potassium.	1.14	0.79
Jan. 15–17 Low potassium.	0.76	1.09	Jan. 23–25 Low potassium.	1.71	1.13
Feb. 1–3 Low potassium.	1.58	1.45	Feb. 1–3 Low potassium.	1.14	0.46
Feb. 4–6 High potassium.	1.35	1.58	Feb. 4–6 High potassium.	1.64	1.42
Feb. 7–10 High potassium.	0.78	0.18	Feb. 7–10 High potassium.	0.76	0.32

TABLE IX.

Pig 5, male. 5,000 cc. of milk fed daily.

Date.	Potassium added.	Potassium excreted.	Sodium excreted.	Chlorine excreted.	Volume of urine.
	gm	gm	gm.	gm.	cc.
Dec. 15		6.86	2.25	4.11	3,700
" 16		7.35	3.07	4.53	4,000
" 17		6.87	3.11	5.84	3,700
" 18	8.49*	14.70	3.15	6.55	3,780
" 19	8.49	14.72	1.74	4.07	3,600
" 20	8.49	15.21	0.82	3.85	3,260
" 21	8.49	16.02	1.30	5.13	3,670
" 22	8.49	15.18	2.06	7.26	3,680
" 23	8.49	11.66	2.12	4.70	3,420
" 24	16.98	22.53	2.38	6.46	3,860
" 25	16.98	22.80	1.74	5.31	3,760
" 26	16.98	22.75	1.46	4.52	3,500
" 27	16.98	22.54	1.29	4.52	3,900
" 28	16.98	22.41	0.97	4.96	3,620
" 29	16.98	22.52	1.57	5.84	3,900
" 30		8.55	2.47	4.43	3,800
" 31		7.15	2.10	5.67	3,500

* $KC_2H_3O_2$

The quantity of potassium does not, however, have a direct bearing on the amount of chlorine in the urine, for during the period December 24 to 29 where the potassium was increased 50 per cent the total excretion was practically the same as during the preceding period. Although this increase in chlorine takes place, the average daily excretion in urine does not equal the amount ingested with 5,000 cc. of milk which would be approximately 7.98 gm.

The results reported here are in harmony with those obtained by Hart and associates (11) where single grain rations were fed to heifers. They report that nearly double the potassium intake had no influence whatever on the sodium chloride intake and that there was no indication that the sodium chloride consumption was regulated or influenced by the amounts of potassium in the feed.

SUMMARY.

The experiments reported here with the pig and employing a starch diet, a synthetic ration, and a milk diet agree with results obtained by Bunge (8) upon the effect of potassium on sodium and chlorine excretion; namely, that a sudden increase of potassium salts in the diet caused an immediate increase in sodium and chlorine excretion during the following 24 hour period.

According to Bunge's data, after the organism had excreted the major portion of the potassium salt, its daily sodium and chlorine excretion during the following 3 days was far less than it was prior to giving the potassium salt. The average daily excretion of sodium and chlorine for the 4 day period after the introduction of potassium phosphate in the food was approximately the same as during the 4 day period preceding the giving of potassium. The data presented in this paper have shown that the sodium and chlorine excretion also decreased when this high potassium intake was continued, similar to what took place after giving one dose of potassium salts. As the high potassium period progressed, a stage was reached where a considerable portion of the ingested sodium chloride did not appear in the urine. The failure of the sodium and chlorine to appear in the urine in quantities equal to the amounts ingested during this apparently retentive period cannot be attributed to the potassium interfering with the ab-

sorption of the sodium. In Tables IV and V where sodium chloride was introduced during the high level of potassium intake, it was 3 or 4 days before the quantities excreted through the kidney became equal to the quantities ingested, yet the quantities of sodium and chlorine excreted finally did equal the amount taken in as sodium chloride. It appears that the animal can distribute its sodium and chlorine as required by the different parts of the body during the absorption of large quantities of potassium, which may at other times cause increased sodium and chlorine excretion. The ability of the animal to control its sodium and chlorine excretion is further shown through the fact that the quantity of potassium taken into the blood at any one time does not determine how much sodium and chlorine will be found in the urine.

Acknowledgment is here made to Professor H. Steenbock who suggested this problem and for his helpful criticisms during the investigation.

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POTASSIUM IN ANIMAL NUTRITION.

II. POTASSIUM IN ITS RELATION TO THE GROWTH OF YOUNG RATS.*

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Chemical analysis of animal tissues gives qualitative information as to what elements are probably required for normal growth and functioning of the animal body, but suggests nothing definite as to minimum mineral requirement. With the success obtained in recent years in growing animals on purified foodstuffs, there is now available a method by which the mineral requirements can be placed on a quantitative basis. Furthermore, the technique developed in the feeding and care of animals together with our improved methods of chemical analysis—all developed since mineral balances were first studied in the animal—now allow studies on the effect of an excess or deficiency of an element to be carried out with great precision.

Osborne and Mendel (1) conducted experiments on the indispensability and interchangeability of inorganic elements in the nutrition of the rat. They report quite normal growth when either potassium or sodium was missing in the salt mixture, but using a salt mixture containing neither potassium nor sodium, failure of growth resulted. From their results it appears that sodium and potassium can substitute for each other in performing some physiological activity necessary to the process of growth. As to what degree, if any, solutions of different salts of equal osmotic pressure are interchangeable is not known. It has not been possible to replace the potassium in the cell by an equiva-

* Part of a thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at the University of Wisconsin.

lent amount of sodium. The organism apparently has a means of keeping the sodium and potassium content of the blood constant in spite of variations in the food supply. Landsteiner (2) in feeding experiments with rabbits found that although two series of animals were fed vastly different relative amounts of alkali, nevertheless, at the end of the experiment the sodium and potassium content of the blood was the same in each case. The author in this investigation found that the blood of both the pig and rat was not altered in its potassium and sodium content when the animals were receiving the diets containing the highest amount of potassium. The proper functioning of the blood undoubtedly depends upon the specific chemical properties exhibited by both the potassium and sodium ions.

Loeb (3) in his work with physiologically balanced salt solutions showed that the ammonium ion acted in cases of antagonism on the egg of *Fundulus* more like the potassium ion than the sodium ion. This corresponds to the fact that in its general chemical behavior the ammonium ion resembles the potassium ion more closely than the sodium ion. Overton (4) showed that frog muscle immersed in isotonic cane-sugar lost its excitability, and that restoration could be brought about by a sodium salt, or in a less degree, by a lithium salt, but not by salts of potassium or ammonium. Mitchell and associates (5) from investigations on the absorption of potassium by animal cells state that their results confirm the theory that the peculiarities in the physiological effects of potassium including the facility with which it is selected in preference to sodium are related to the electronic structure of the potassium ion as compared with that of similar ions.

In order to study further the effect of a minimum potassium diet on growth and to determine if sodium could replace potassium in its physiological function necessary to the process of growth, the second part of this investigation was undertaken. Rations containing a high ratio of potassium to sodium were also used to ascertain, this time, the influence of a high potassium intake on growth. The rat was chosen for this work because of the necessity of employing purified foodstuffs, which, due to the labor and expense of preparation, can only be used in small quantities.

EXPERIMENTAL.

Naturally in beginning the study on the minimum requirement for potassium in the growth of the rats it is necessary that the components of our ration be prepared so as to contain the least possible quantity of potassium. If by so doing, this element is reduced to such a low level that a physiological disturbance results from this deficiency of potassium in the ration, then definitely increasing quantities of potassium may be added, and from these results information will be obtained on the quantitative potassium requirement for growth and normal well being of the animal.

While most of the constituents of the ration can be used in a highly purified form as obtained by water or acid extraction, yeast used as the source of the water-soluble vitamine cannot be purified in this way.

The introduction of yeast into the otherwise artificially prepared food mixture also carries with it certain quantities of potassium. The yeast available contained 2.1 per cent of potassium. This yeast when fed at a 2.0 per cent level would introduce 0.42 gm. of potassium per kilo of ration. As it was desired to avoid such a large quantity of potassium, analytical methods were sought by which this potassium could be removed from the necessary constituents without destroying the latter.

Preliminary trials showed that water would easily extract the potassium salts from this yeast. As both the potassium and the water-soluble vitamine are therefore taken out by water extraction the possibility of removing the potassium from this extract presented itself. To accomplish this, the principle was made use of that potassium-acid tartrate is insoluble in 50 per cent alcohol solution, leaving a solution rich in vitamine and containing only a slight excess of the comparatively innocuous tartaric acid. Accordingly, the following procedure was adopted.

100 gm. quantities of yeast were extracted with 600 cc. of water in 300 cc. portions, the solution was separated from the residue by centrifuging each time. The residue was dried over steam coils and saved for later use. The solution was concentrated over the steam coils to one-half of its original volume and 10 gm. of tartaric acid were added. Alcohol (95 per cent) was added to twice the volume and this solution was allowed to stand 24 hours

and then filtered. Analysis of this solution showed that 96 per cent or more of the potassium had been removed.

In order to determine which portion was richest in the water-soluble vitamine and at what levels the residue or extract should be fed in order to obtain normal growth with the artificial food mixture, a series of experiments was carried on.

To incorporate this extract in the ration a definite quantity equivalent to a known amount of yeast was evaporated on dextrin. This was then fed in the rations in different units so as to supply various amounts of water-soluble vitamine. The residue, corresponding to 70 per cent by weight of the original yeast, was also fed at different levels as shown in Charts 1 to 9. The rats unless otherwise indicated were allowed to run on shavings, and were given free access to distilled water and to the desired ration.

Charts 1 to 9 show the amount of the yeast preparations required to supply a sufficiency of the water-soluble vitamine. The small amounts of tartaric acid in the ration apparently were rendered harmless by the organism, at least the animals did not appear to be suffering from tartrate nephritis as Underhill (6) was able to demonstrate when dogs and rabbits were injected subcutaneously with comparatively larger amounts of sodium tartrate in solution. From these results it was decided to feed the extract in quantities equivalent to 10 per cent of the original yeast in our minimum potassium series reported later on.

In order to arrive at the approximate minimum quantity of potassium necessary for normal growth, groups of rats were fed on a ration in which the potassium was omitted from the salt mixture. Salt Mixture 32 as given below and used by Steenbock afforded an easy means of omitting the potassium as this salt mixture contains potassium only in the form of dipotassium-acid phosphate which could be left out of the salt mixture—sufficient phosphorus still remaining in the form of sodium and calcium phosphates and the casein used. Another group of rats was fed the same ration except that an equivalent amount of sodium phosphate was substituted for the potassium phosphate. Another group received Salt Mixture 32, complete as a control. Charts 12, 13, and 14 show the results of these trials.

NaCl	40
$MgSO_4$	62
Na_2HPO_4	41
K_2HPO_4	223
$Ca_2H_2(PO_4)_2 \cdot 4H_2O$	223
Ca lactate	57
Ferric citrate	27

Having reduced the increment of growth by employing a potassium-free salt mixture in the ordinary synthetic ration, another ration containing yeast extract as previously described plus especially prepared casein, washed starch, and washed agar was fed to three additional groups of animals. These three groups of animals were placed on screens so that they would have no opportunity to eat their feces, and shavings were used as litter.

The growth curves of these trials are given in Charts 25 to 27. Though the effect of a ration low in potassium is readily seen in the growth curves the rats receiving this ration showed no other anatomical abnormalities. However, they were very alert and appeared to crave for some constituent not present in the ration, and though feed was always present in their feeding cup, introduction of an additional quantity caused them to consume portions of the ration in a greedy manner. They showed perfect control in all actions of the body. The failure of the animals to respond to potassium when introduced into the ration was not expected. Unless 6 weeks were not sufficient time for the animal to readjust itself to the change in diet, this dietary deficiency in early life may have interfered permanently with the development of the animal organism. From Chart 10 it can be seen that the animals responded to increased potassium in the diet at the end of the first 4 weeks. However, after a period from 10 to 14 weeks they ceased to grow, gradually became afflicted with edematous eyes and respiratory trouble, and died. As special precautions were taken to supply all the necessary dietary constituents for growth and normal well being, the death of these animals must have been primarily due to a deficiency of potassium during early life.

The total potassium content of the ration employed in the minimum potassium series contained about 0.037 gm. of potassium per kilo, while the ration in which the potassium phosphate was omitted contained approximately 0.55 gm. of potassium per kilo

of ration. This difference in potassium content is nearly all accounted for by substituting the potassium-free yeast extract for the whole yeast. As the charts show plainly the effects of feeding potassium at these levels, a further study was made on the growth of rats by introducing definite increasing quantities of potassium into the low potassium ration.

The results so obtained are given in Charts 15, 16, and 17. Apparently, from these data the minimum potassium requirement lies somewhere between 0.55 and 1.44 gm. per kilo of the ration here employed.

The results obtained above with a ration containing 0.05 per cent potassium are not in accord with those reported by Osborne and Mendel where they obtained fairly normal growth with a ration containing about 0.03 per cent potassium. As they employed a ration high in fat and, therefore, one far different from the ration used by the writer, and as the potassium requirements may vary with the other constituents of the ration the writer attempted to duplicate their feeding trials as reported.

The results obtained on this low potassium ration are shown in Charts 28 and 29. In general they do not confirm the findings of Osborne and Mendel. However, the failure in this case to obtain normal growth with their complete ration caused by a low content of water-soluble vitamine in the yeast as brought out in comparison of Charts 30 and 31 does not present the opportunity to observe in a satisfactory manner the results of a potassium-free salt mixture in their ration.

In the study of the effect of high potassium to sodium on growth the disodium-acid phosphate and sodium chloride were omitted from the salt mixture and equivalent amounts of dipotassium phosphate and potassium chloride were substituted. While this low sodium ration was fed to one group other groups received the same ration plus definite increasing quantities of sodium phosphate. In addition three groups of rats were fed the same quantities of sodium phosphate plus 30.0 gm. of potassium citrate per kilo in order to study any effects that an excessively high potassium-sodium ratio might have.

Results obtained on low sodium feeding are expressed in Charts 18 to 20. The satisfactory growth that resulted from leaving sodium out of the salt mixture is not surprising as the ration con-

tained about 0.07 per cent of this element. The curves representing successful growth during the high potassium intake are given in Charts 22, 23, and 24.

Neither the normal sodium nor potassium content of the blood was altered by feeding a ration high in potassium. Male rats (Nos. 41 and 42), after having been on the complete ration for 18 weeks were given a ration in which the normal sodium content of the salt mixture was replaced by potassium and an additional 60 gm. of potassium citrate was added to a kilo of the ration. After 3 weeks blood was obtained by severing the carotid artery while the animal was under the influence of an anesthetic. Analysis showed 0.187 per cent potassium and 0.178 per cent sodium by weight. Blood from Animals 53 and 55, which had received the complete ration, contained 0.188 per cent potassium and 0.170 per cent sodium.

SUMMARY.

The experiments reported here show that the growth of rats can be greatly retarded by reducing the potassium content of the ration below a certain level, approximately 0.1 per cent. During the experimental period on this low potassium diet no other distinct physiological abnormalities made their appearance as a result of this nutritive deficiency. Whether the animal would respond to increased potassium in the diet seemed to depend at what growth stage the potassium phosphate was given. Response was obtained with one group of animals during the 4th week, while two other groups did not respond when either potassium phosphate or potassium chloride were added to the feed during the 11th week. Though response was obtained as above mentioned, it was not permanently successful. Cessation of growth finally took place, with death supervening. While failure of growth was readily apparent in feeding young rats a ration deficient in potassium, no other physiological disturbance manifested itself during the period on low potassium. Only when potassium was given did other harmful symptoms later appear. This indicates that inadequate potassium during the early development of the organism may not only prevent the growth of body tissue but also cause abnormal physiological changes which make themselves apparent later on.

The failure to obtain normal growth by substituting sodium for the potassium does not support the view that sodium may replace potassium in the physiological functions that it performs in the phenomenon of growth. A ration in which the ratio of potassium to sodium was 14:1 had no deleterious effect on the growth of young rats. The normal potassium and sodium content of the blood was not altered by feeding a ration high in potassium.

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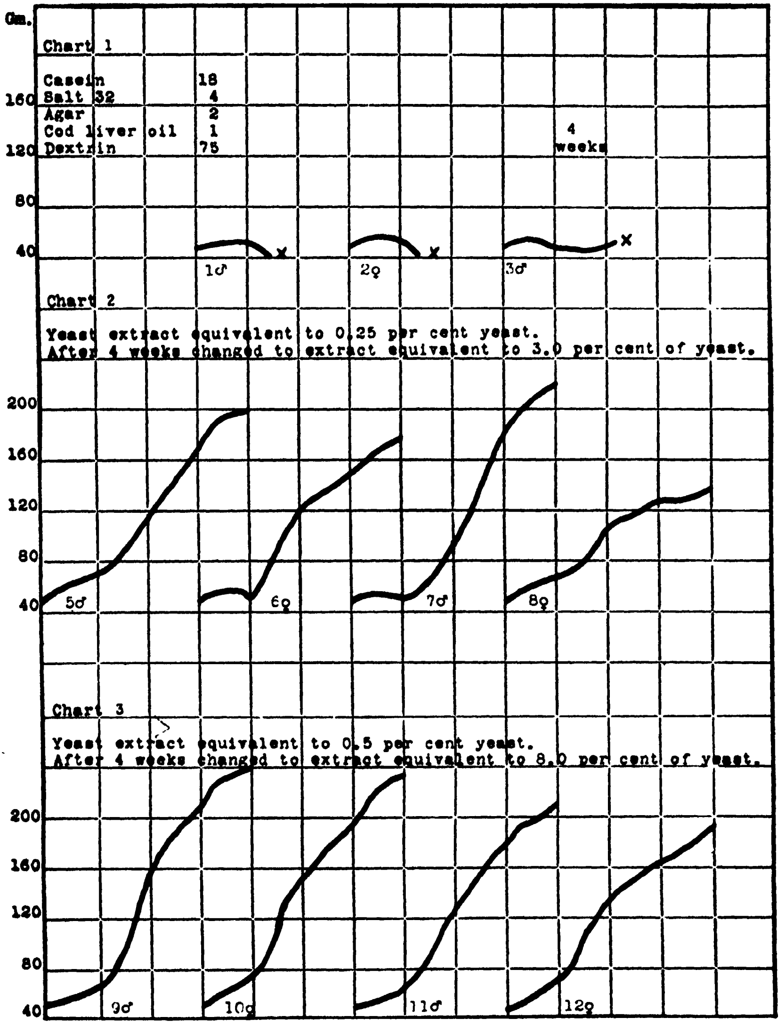
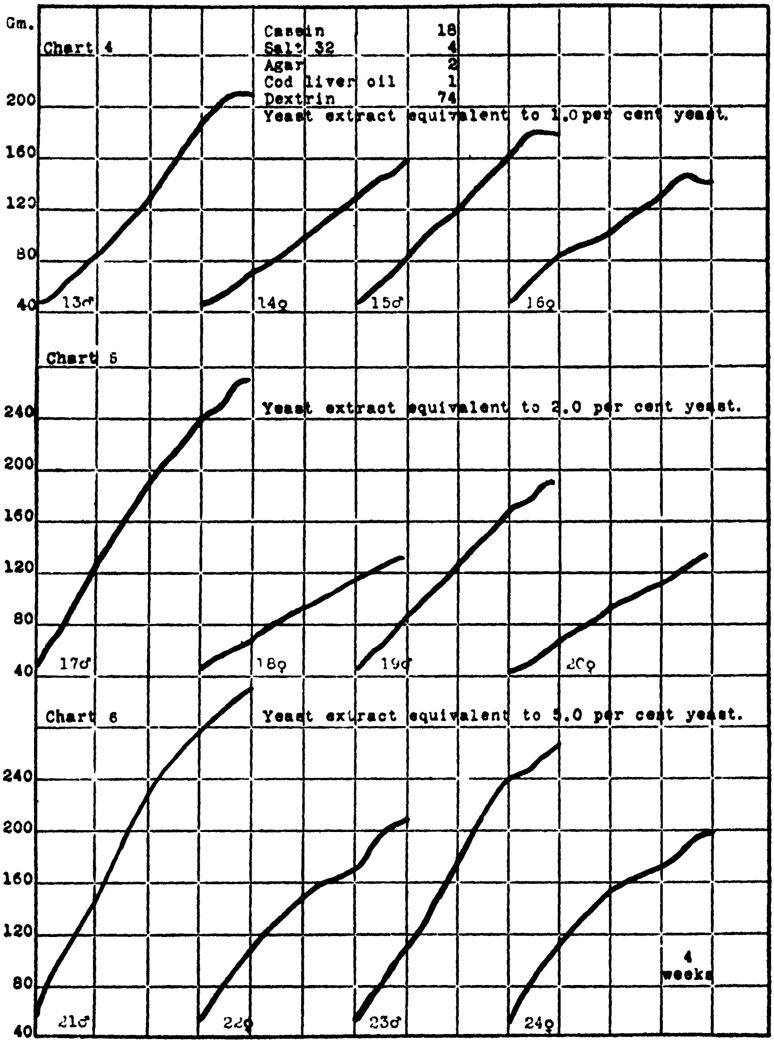
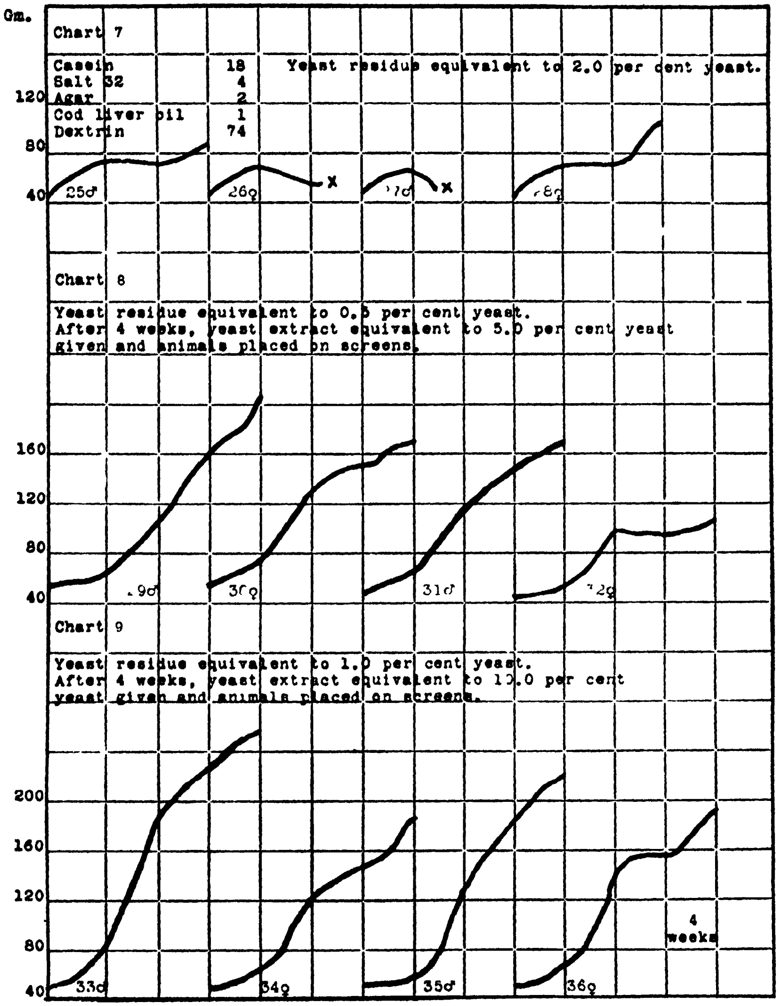


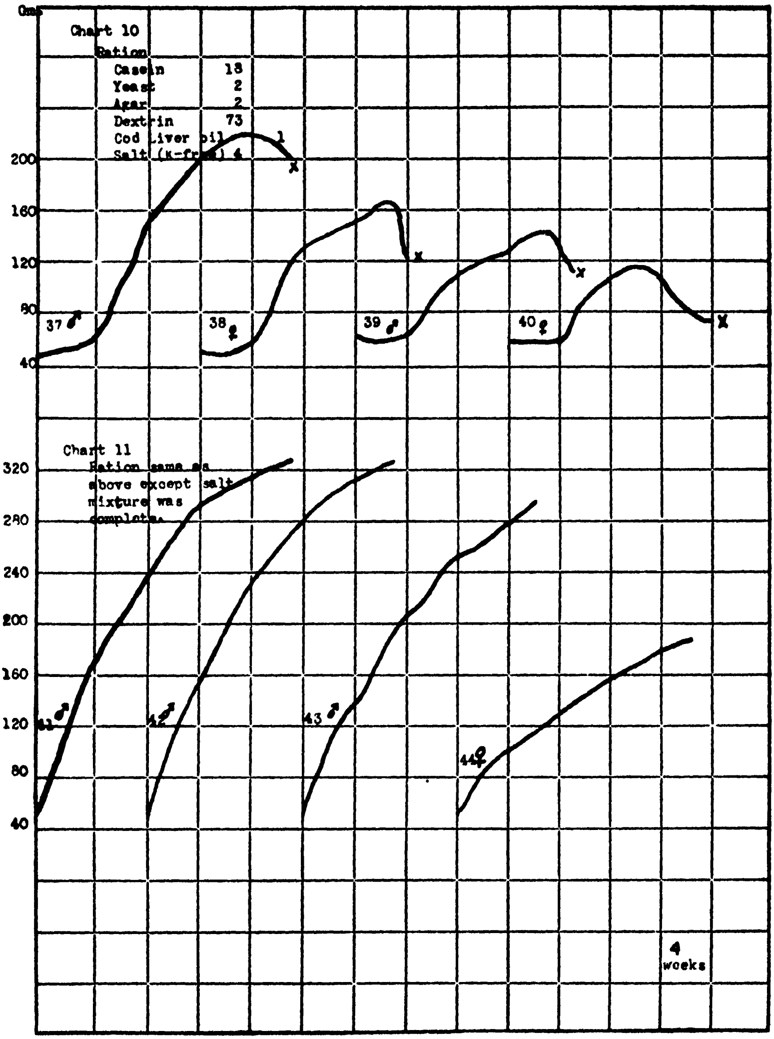
Charts 1, 2, and 3. Young rats did not grow on the ration containing no yeast, death finally occurring. The effect of feeding the extract at 0.25 and 0.50 per cent levels and the response after introducing yeast extract equivalent to 3.0 and 8.0 per cent of the original yeast is readily seen.



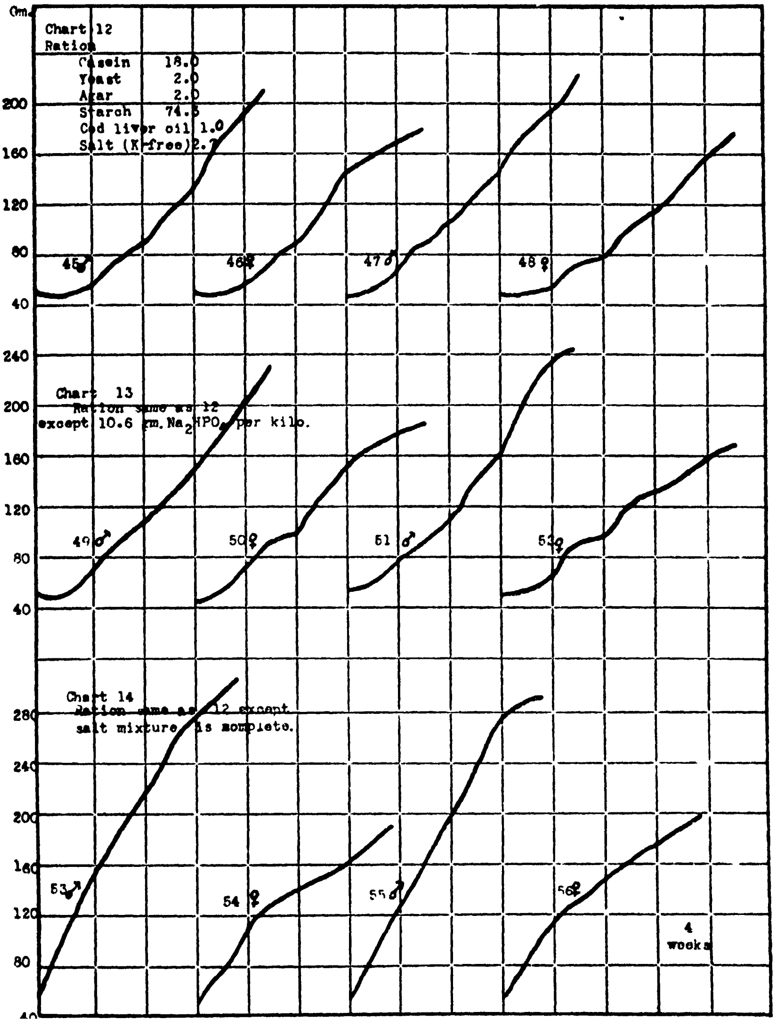
CHARTS 4, 5, and 6. The above curves show the results of feeding yeast extract equivalent to 1, 2, and 5 per cent of yeast as the source of water-soluble vitamine. Feeding the extract at a 5 per cent level apparently suffices for normal growth.



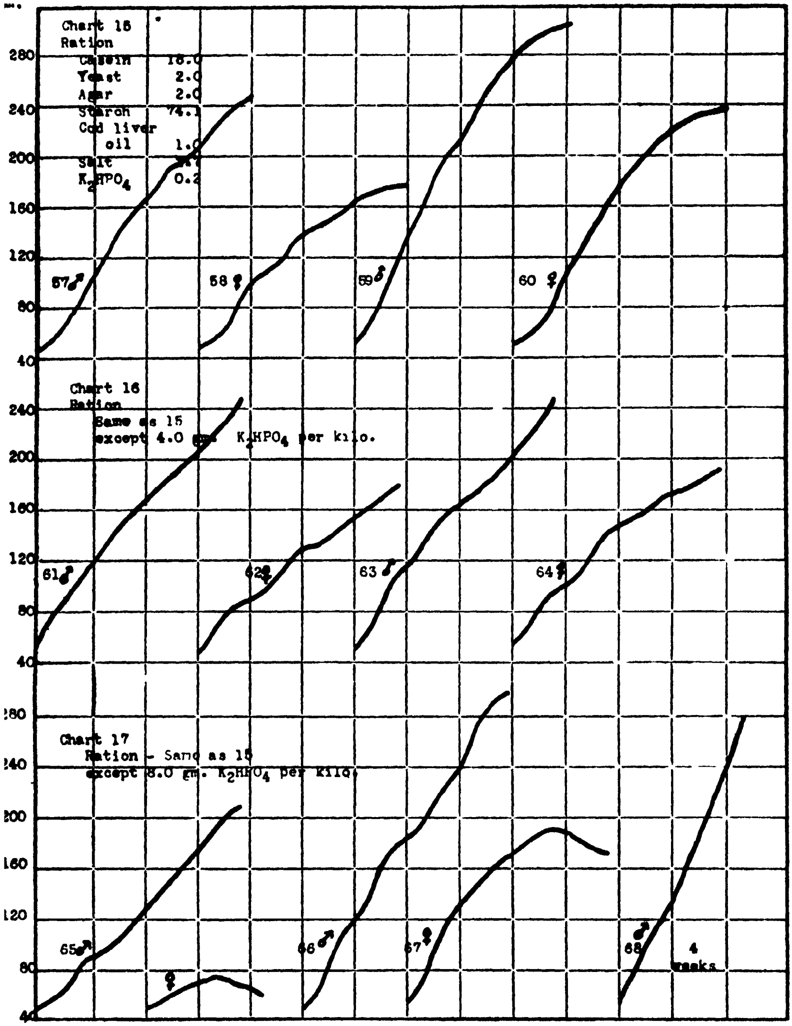
Charts 7, 8, and 9. The small amount of water-soluble vitamine retained in the yeast residue is shown in the above curves. Continued feeding of the yeast residue at a 2 per cent level resulted in growth failure and death. Satisfactory growth was obtained when rats were placed on screens and yeast extract equivalent to 10 per cent of yeast was added to the ration.



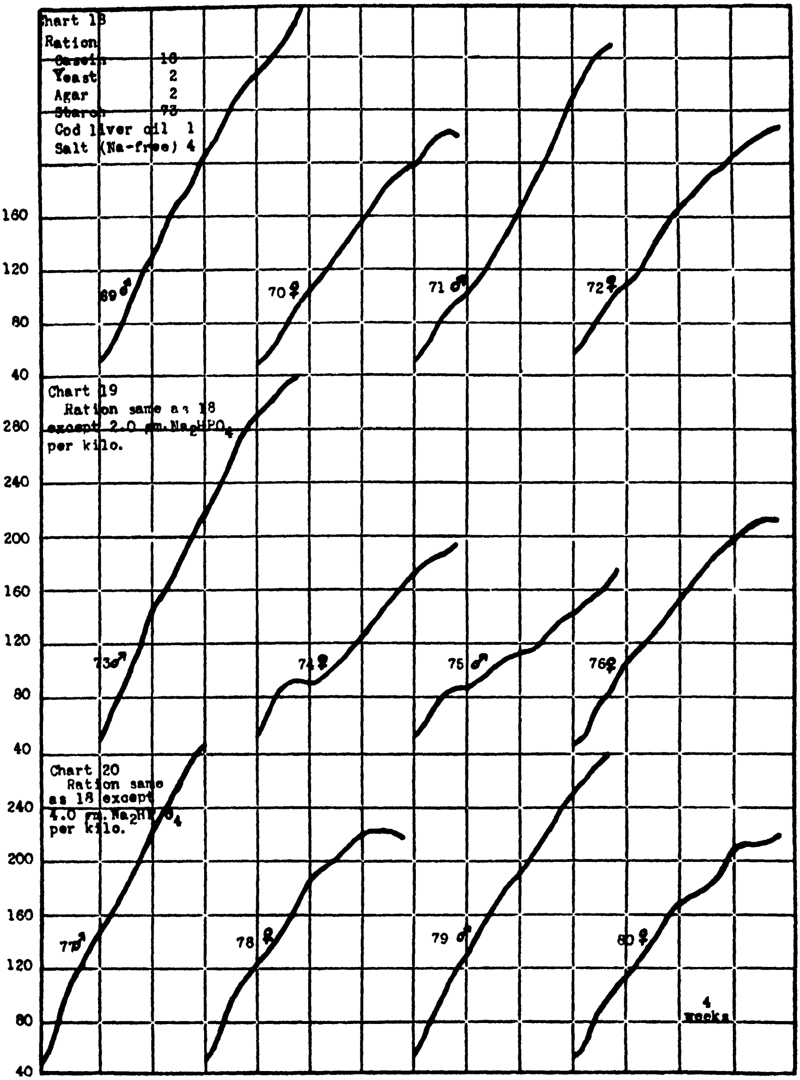
CHARTS 10 and 11. Compared to the control ration there was practically no gain in weight during the first 4 weeks when potassium phosphate was omitted from the salt mixture. Introducing potassium phosphate apparently brought about an immediate response but later on all the animals in Group 10 (Chart 10) became afflicted with edematous eyes and respiratory trouble, followed by death. Special precautions in the preparation of the diet and the satisfactory growth with the control group strongly indicates that this unsatisfactory condition was due to deficiency of potassium in the early development of the organism.



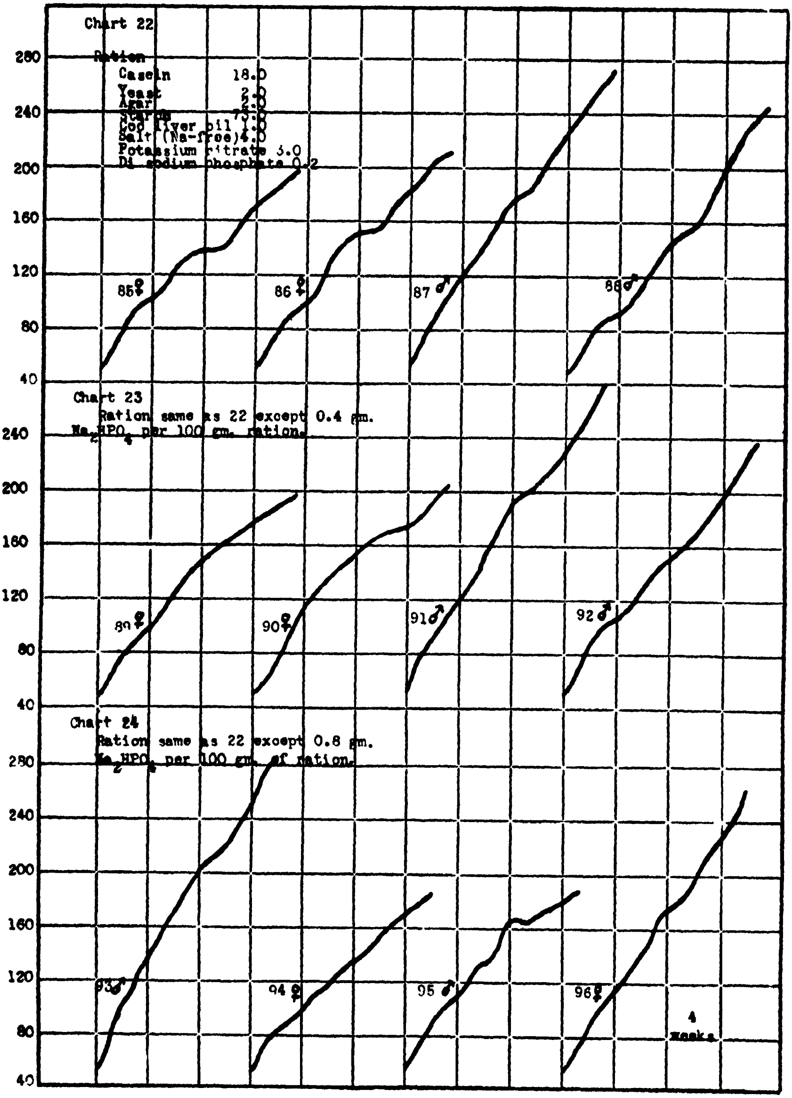
CHARTS 12, 13, and 14. The growth curves show the effects of a deficiency of potassium in these rations brought about simply by not introducing the potassium into the salt mixture. Substitution of equivalent sodium phosphate for the potassium phosphate removed does not completely replace the potassium in the physiological function it performs in growth.



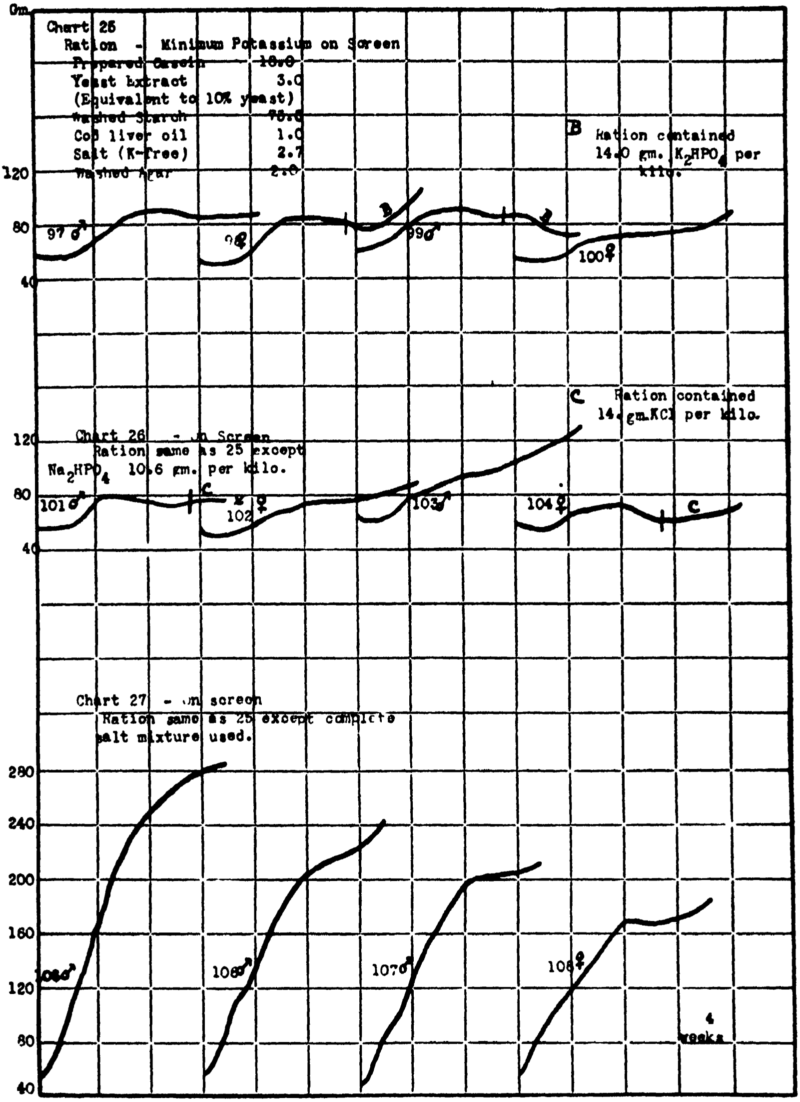
CHARTS 15, 16, and 17. Introducing 2.0 gm. of K_2HPO_4 into a kilo of the low potassium ration caused an increase in growth so that the curves correspond very well to the normal growth curves. Additional quantities of potassium phosphate have brought about no further increase. Rat 66 was anesthetized and post mortem showed abnormally large and congested lungs.



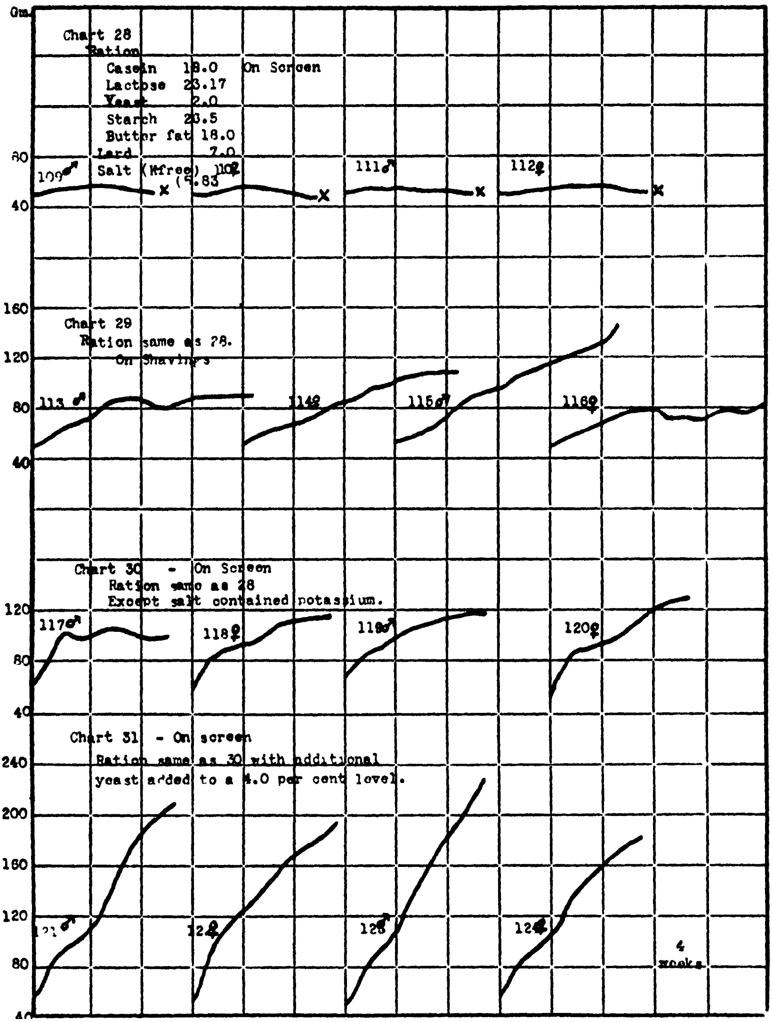
CHARTS 18, 19, and 20. Removing sodium from the salt mixture and replacing it with potassium did not interfere with the growth of young rats. No sodium deficiency was apparent, neither did this high ratio of potassium to sodium prove deleterious in any way.



CHARTS 22, 23, and 24. Successful growth was obtained where an additional 30.0 gm. of potassium citrate was added to the low sodium rations.



CHARTS 25, 26, and 27. On rations containing minimum potassium there is a decrease in the weight of young rats at first, followed by exceedingly slow growth. Sodium did not replace potassium to any extent as far as the growth curves show. At the end of 11 weeks, two animals in each group were given potassium as chloride in one case and phosphate in the other without any general response.



Charts 28, 29, 30, and 31. Where potassium was omitted from the salt mixture in the above ration the animals failed to grow and finally died. Group 30 (Chart 30) receiving potassium grew slowly but did follow the normal growth curve. Similarly, the group receiving no potassium in the salt mixture, but allowed to run on shavings, grew to a certain extent. Surmising that there was a deficiency of the water-soluble vitamine in this ration of high fat content, the yeast content was increased to 4 per cent in one ration, producing results as indicated in Chart 31. The charts show the deficiency of potassium and the water-soluble accessory in the rations fed Groups 28, 29, and 30.

THE OCCURRENCE OF COPPER AND ZINC IN CERTAIN MARINE ANIMALS.*

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At a very early date the fact that blood and tissues of certain marine animals turned a dark bluish color when exposed to the air caused investigations to be made. In 1817 Erman, according to Kobert, made a study of the blue dye of snake blood. Then followed work on the blood of marine animals by Caries in 1824, Wharton in 1846, Genths in 1852, and Schlossberger in 1857. Copper was found in the blood of *Eledone* and *Helix pomatia* by Harless in 1847. In the plasma, the copper was reported in combination with a protein, but was not found in the protein of the corpuscles. The protein copper compound had the power to unite with oxygen, thus forming a substance which appeared analogous to oxyhemoglobin. Fredericq in 1878 named the protein containing copper in the blood of the *Octopus vulgaris*, hemocyanin.

Hemocyanin has been found in certain early epochs by paleontologists. It may have been a matter of evolution that in the early periods hemocyanin was a constituent of the blood as well as the hemoglobin, since both are oxygen carriers. Henze (1901) showed a similarity in many of the properties and reactions between hemocyanin and hemoglobin.

The arterial blood of many invertebrates is colorless, but by taking up loosely bound oxygen it is converted into the oxyhemocyanin. According to Henze (1901) hemocyanin combines with about 0.4 gm. of oxygen per gram of hemocyanin. Griffiths (1890) found that the hemocyanin of different marine animals would take up varying amounts of oxygen, nitrogen, and carbon dioxide gases. The nitrogen was simply dissolved in the blood, but the carbon dioxide and oxygen were partly dissolved and partly in a state of loose chemical combination with certain constituents of the blood. Investigators, such as Lankester (1872), Halliburton (1885–86), Dubois (1900), Alsberg and Clark (1910), and Philippi (1919), who made a very careful study of hemocyanin for many species, report that it differs in composition as well as in its combining power with oxygen. From the investigations of Dhéré (1900 to 1919), who examined the blood of different types of marine animals it was found that the oxygen-combining power was

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dependent upon the amount of copper these hemocyanins contained. Mendel and Bradley (1905), have shown that the more active an animal is, the higher the copper content, and *vice versa*. This is to be expected, since with the active animal there should be more rapid respiratory changes.

Copper has been found in the tissues, liver, intestines, and muscles of oysters. According to investigators such as Willard (1908) and Hiltner and Wichmann (1919), oysters may carry enough copper to give a greenish color, as well as a metallic taste. Muttkonski (1921) made a study of the copper content of plants and insects, such as daddy-long-legs, centipedes, bees, and ants, and obtained a positive reaction for copper, but in varying amounts.

Zinc seems to be often associated with copper. In 1877, Sachester and Bellamy found zinc in human liver, ox liver, eggs, wheat, and vegetables. According to Dieulafait (1880), paleontologic schists, Silurian and Devonian deposits, as well as fossils, contained zinc. He analyzed the sea water of the Mediterranean and reported 2 mg. per cubic meter. In the liver and blood of *Sycotypus canaliculatus*, and *Fulgur carica*, Bradley (1904) and Mendel and Bradley (1905) obtained zinc. Certain Tortugas marine forms were examined by Phillips (1917); in all of which he showed the presence of zinc, except in the crayfish blood.

In 1919 Hiltner and Wichmann made a study of the zinc in the oysters, while Delezenne, in the same year, worked with snake venom. According to Delezenne, the zinc in the blood of animals occurs for the most part in the leucocytes, and only a small amount is present in the plasma. The zinc in snake venom is in combination with a protein. Jamieson (1919) determined copper and zinc in gelatin, Rose and Bodansky (1920) and Bodansky (1920) made a study of the zinc content of several different marine animals, finding that the zinc in the oyster was rather uniformly distributed through the tissues.

Since, it appears that both copper and zinc play an important physiological part in the life of these marine animals, and because the above investigations were made for the marine organisms of the Atlantic coast, it was thought of interest to investigate the copper and zinc content of some of the marine animals, including certain invertebrates, whale and sea-lion, of the Pacific coast.

In order to have a fair basis for a comparison of marine animals for the Atlantic and Pacific coasts, Rose and Bodansky's (1920) method was used for the determination of copper, and Bodansky's (1920) modification of Birckner's (1919) method was followed for zinc. Great care was taken to follow closely each step of these methods, results being tabulated in Table I. Most of the material worked with was collected fresh and analyzed as soon as possible. Shrimps, oysters, crab, salmon, and clams were obtained

through a local market, and also from San Francisco markets. These were obtained by the markets fresh from the waters of the Pacific coast. The cryptochiton, sea anemone, starfish, abalone, sea-urchin, and mussel were collected at Half Moon Bay. The sea-lion (*stelleri*) came from Ano Nuevo Island, 1 mile off the coast, near Pescadero. The whale was obtained from the whaling station near Monterey.

The specimens were thoroughly minced and mixed before sampling. Where possible the entire animal was used. Mussels, clams, and oysters were shucked. In some cases the shell and protective coverings were run for copper and zinc.

EXPERIMENTAL.

The reagents used were carefully tested for both copper and zinc, and were found to be free from these metals.

Determinations of copper and zinc were made for samples of sea water obtained from the ocean between the main land near Pescadero and Ano Nuevo Island. In these samples were found not a trace of copper and 0.00194 mg. of zinc per kilo of sea water.

Dieulafait (1880) found 2 mg. of zinc per cubic meter for the Mediterranean waters; Hiltner and Wichmann (1919) reported no zinc or copper in a sample of sea water taken 1 mile off-shore from West Sayville, New York, while Bodansky (1920) found a maximum for the sea water 30 feet from the Galveston shore to be 0.0073 mg. of copper per kilo, and 0.14 mg. of copper per kilo of water.

Table I shows the results of copper and zinc from the analyses of fifteen different marine animals, and one species of land slug. For the most part the results show the presence of one, or both, of these metals in varying amounts. Of the mollusks, the oysters, shrimps, blood of cryptochiton, and yellow slug showed the greatest amount of copper. The largest amount of copper found in any one of the species analyzed was 19.60 mg. per kilo in the shrimps, while the least amount found was just a trace in the mussel shell, and spleen and liver of the sea-lion. The internal shell of the cryptochiton, and the whale and clam, did not give any copper for the amount taken for analysis. The results on clams correspond to those found by Rose and Bodansky (1920).

TABLE I.

Amount of Copper and Zinc in Certain Marine Animals.

Sample No.	Animal.	Amount analysed.	Copper in fresh material.		Zinc in fresh material.		Remarks
			Per sample	Per kilo.	Per sample	Per kilo.	
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	
	Cœlenterata.						
	Actinoza metridium.						
SA-I	Sea anemone.	100 00	0.10	1 00	1 00	10.0	1 large and 2 small animals; ground.
SA-II	" "	100.00	0 10	1 00	1.10	11.0	
	Echinodermata.						
	Asterias orcacea.						
SF-BI	Starfish; black.	100 00	0.30	3 00	2 00	20.0	1 animal.
SF-BII	" "	100.50	0.33	3.28	1.10	10.9	1 " cut up and sampled.
SF-BIII	" "	100.00	0.29	2.90	2.00	20.0	
SF-BIV	" "	181.70	0.32	1.76	4.00	22.0	
SF-YI	" yellow.	127.50	0.30	2.35	2.10	16.4	1 entire animal.
SF-YII	" "	100.00	0 22	2.20	1.50	15 0	1 " "
	Patari nuniaba.						
SF-RI	Starfish; red.	53.10	0.10	1.88	1.00	18 8	1 " "
SF-RII	" "	53.50	0.10	1.86	1.00	18.6	1 " "
SF-RIII	" "	65.70	0 15	2 20	1.30	19.8	1 " "
	Puperatus strongylocentrotus.						
SU-I	Sea urchin.	93.00	0.16	1.72	0.20	2.15	Several small animals without shell.
SU-II	" "	91.80	0.15	1.63	0.20	2 07	

	Mollusca.						
	Limas maximus.						
S–I	Yellow slug.	100.00	0.12	1.20	3 00	30.0	Collected sample; young snails.
S–II	" "	100.00	0.20	2.00	3.00	30.0	
S–III	" "	100.00	0.61	6.10	3 00	30 0	Collected sample; older snails.
S–IV	" "	100.00	0.50	5.00	3.00	30.0	
S–V	" "	151.36	0.75	4.95	5.00	33.0	Collected about 6 months later.
S–VI	" "	189.30	0 96	5.12	6.10	33.0	
O–I	Oysters (Eastern).	25.00	0.75	30.00	4.25	170 0	1 oyster shucked.
	Ostrea lurida.						
O–II	Oysters (California.)	50.00	0.33	6.60	4.00	80 0	Oysters shucked; from San Francisco.
O–III	" "	49.10	0.25	5.10	4.00	81.0	
O–IV	" "	100.10	0.57	5.00	10 00	99 0	Meat of 200 oysters bought 5 weeks later from market.
O–V	" "	128.07	0.70	5.40	12.50	97 4	
O–VI	" "	100.00	0.13	1.30	1.50	15.0	Liquid of 200 oysters bought at same time.
O–VII	" "	125.50	0.18	1.40	1.50	15 0	
O–VIII	" "	156.00	0.67	4.30	15.00	96.1	Meat and liquid of 100; bought 3 weeks later.
O IX	" "	147.70	0.25	1.70	4.20	28.44	
	Ensis americanus.						
C–I	Clams, pismo.	152.00	0.00	0.00	2.30	15.09	1 animal shucked.
C–II	" "	135.90	0.00	0 00	2 00	14.72	1 " "
	Venus kennicottii.						
C–III	Clams, little neck.	58.90	0.00	0.00	0.30	5.09	1 lb. shucked.
	Mylibus californica.						
M–I	Mussel; meats.	40.32	0.135	3.34	2.00	49.5	Young from Half Moon Bay; shucked.
M–II	" "	90.00	0.34	3.77	3.80	40.22	
M–III	" shell.	100.00	Trace.	Trace.	0.00	0 0	Monterey mussels; older; shucked.
M–IV	" "	100.00	"	"	0 00	0.0	

TABLE I—*Continued.*

Sample No.	Animal.	Amount analysed.	Copper in fresh material.		Zinc in fresh material.		Remarks.
			Per sample.	Per kilo.	Per sample.	Per kilo.	
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	
	Haliotis crackerodia.						
AB–I	Abalone.	100.00	0.08	0.80	2.50	25.0	Sample had been kept in alcohol several weeks.
AB–II	"	100.00	0.08	0.80	2.50	25.0	
AB–III	"	100.00	0.08	0.80	2.50	25.0	
AB–IV	"	100.00	0.08	0.80	2.50	25.0	White steak part.
AB–V	"	140.80	0.11	0.78	2.90	20.6	Fresh animal; entire, without shell.
	Cryptochiton stelleri.						
Cry–I	Cryptochiton; foot.	30.33	0.05	1.64	0.50	16.49	
Cry–II	" mantle.	100.00	0.405	4.05	1.00	10.0	1 small animal.
Cry–III	" "	50.00	0.20	4.00	0.20	4.0	
Cry–IV	" "	50.00	0.20	4.00	2.00	40.0	Minced mantles of 10 cryptochiton.
Cry–V	" "	50.00	0 20	4.00	2.00	40.0	
Cry–VI	" "	50.00	0 20	4.00	2.00	40.0	
CB–I	" blood.	100.00	0.50	5.00	0.10	1.0	Blood of 10 animals; mixed and sampled.
CB–II	" "	100 00	0.505	5.05	0.10	1.0	
CB–III	" "	100.00	0.545	5.45	0.10	1.0	
B–I	" internal shell.	50.25	0.00	0 00	0.00	0 0	Shell dissected from 10 animals and sampled.
B–II	" " "	50.55	0 00	0.00	0 00	0.0	
B–III	" " "	40.33	0 00	0.00	0 00	0.0	
St–I	" digestive organs.	100 00	0.05	0.50	0.30	3.0	Organs from 10 animals.
St–II	" " "	100 00	0.06	0.60	0.30	3.0	
Ms–I	" foot (muscle).	50 00	0 05	1.00	0.90	18.0	Pedal muscle or foot of 10 animals minced and sampled.
Ms–II	" " "	50 00	0 06	1.20	1.00	20.0	
Ms–III	" " "	50 00	0.05	1.00	0.90	18.0	

	Arthropoda.						
	Crustacea.						
	Palæmonetis vulgaria.						
Sh-I	Shrimps.	50.20	0.185	3.68	1.20	23.7	Fresh shrimps with protective covering from San Francisco.
Sh-II	"	50.30	0.94	18.60			
Sh-III	"	50.00	0.98	19.60	1.10	22.0	
Sh-IV	"	100.20	1.80	17.96	2.40	23.7	Fresh shrimps; obtained 6 weeks later.
Sh-V	"	100.00	1.86	18.60	2.38	23.8	
	Hermit cupagurus.						
Cr-I	Crab.	24.18	0.05	2.06	2.25	93.6	Without shell; from Half Moon Bay.
	Callinectes hastatus.						
Cr-II	Crab.	60.30	0.13	2.13	0.70	11.5	Legs only of 1 large edible crab.
Cr-III	"	69.90	0.14	2.06	3.00	44.3	
Cr-IV	" except shell.	100.00	0.35	3.50	2.50	25.0	
Cr-V	" " "	100.00	0.35	3.50	2.40	24.0	Shell removed carefully from 1 crab; weight 606 gm.; rest minced, mixed, and sampled.
Cr-VI	" " "	108.60	0.39	3.60	2.50	25.0	
Cr-VII	" shell only.	100.00	0.20	2.00	2.50	25.0	
Cr-VIII	" " "	100.00	0.22	2.20	2.40	24.0	
Cr-IX	" " "	68 70	0.10	1.45	0.50	7.0	
	Teleostomi.						
	Oncorhynchus tschawytscha.						
Sa-I	Salmon.	100.00	0.40	4.00	0.80	8.0	2 slices of fresh salmon from local market; bone removed.
Sa-II	"	100.00	0.40	4.00	0.80	8.0	
	Mammals.						
	Stelleri.						
SSM-I	Sea-lion; muscle.	50.00	0.10	2.00	2.60	52.0	Sea-lion from Ano Nuevo Island near Pescadero.
SSM-II	" " "	50.00	0.10	2.00	2.60	52.0	
SSM-III	" " "	50.00	0.10	2.00	2.80	56.0	

TABLE I—*Concluded*.

Sample No.	Animal.	Amount analyzed.	Copper in fresh material		Zinc in fresh material.		Remarks.
			Per sample.	Per kilo.	Per sample	Per kilo.	
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	
SSM–IV	Sea-lion; muscle.	100.00	Trace.	Trace.	5.00	50.0	2nd sea-lion.
SSM–V	" "	100.00	"	"	5.00	50.0	
SIS–I	" spleen.	50.00	"	"	0.50	10.0	All samples minced and sampled.
SIS–II	" "	50.00	"	"	0.60	12.0	
SIS–III	" "	100.00	"	"	1.00	10.0	Samples from 2nd sea-lion 3 weeks later.
SIS–IV	" "	102.10	"	"	1.20	11.75	
SIL–I	" liver.	50.00	"	"	2.40	48.0	
SIL–II	" "	50.00	"	"	2.40	48.0	
SIL–III	" "	122.20	"	"	5.80	48.2	
SIL–IV	" "	102.20	"	"	4 90	48.0	2nd sea-lion.
SIL–V	" "	100.20	"	"	0 50	49.0	
SIB–I	" blood.	100.00	None.	None.	0.01	1.0	
SIB–II	" "	100.00	"	"	0.01	1 0	
SIBi–I	" bile.	50.00	0.18	3.60	0.12	2.4	
WS–I	Sperm whale; liver.	100.00	None.	None.	4.00	40.0	
WS–II	" " "	100.00	"	"	4.00	40.0	Fresh samples.
WS–III	" " "	100.00	"	"	4.00	40.0	
WM–I	" " "	100.00	"	"	4.00	40.0	
WM–II	" " "	100.00	"	"	4.00	40.0	
WM–III	" " "	100 00	"	"	4.00	40.0	
	Sea water.	*cc.*					
SW–I	Sea water; near shore.	125	"	"	0 05	0.39	1 liter from near Pescadero.
SW–II	" " " "	250	"	"	0.10	0.39	
SW–III	" " 1 mile out.	500	"	"	0.01	0.002	
SW–IV	" " 1 " "	500	"	"	0.01	0.002	Per liter.

The California oyster, *Ostrea lurida,* did not seem to carry as large an amount of copper as the Eastern oyster, according to the analysis of Bodansky (1920). Samples of oysters (see Sample O-I) gave 6.6 mg. per kilo, while Sample O-VIII shows 4.3 mg. per kilo. The liquid drained from the oyster ran from 1.3 to 1.7 mg. per kilo, or for the eight samples analyzed, the average would be 3.9 mg. per kilo of tissue and liquid. This would indicate that some of the copper of oysters is to be found in the liquid.

TABLE II

Average Amount of Copper and Zinc in the Different Animals Examined.

Animal	No of sample	Copper Average for samples	Zinc Average for samples
		mg per kilo	*mg per kilo*
Sea anemone .	2	1 00	10 50
Starfish (black)	3	2 710	20 72
" (yellow)	2	2 273	15 70
" (red)	3	1 986	19 06
Sea urchin	2	1 675	2 11
Yellow slug	6	4 06	31 00
California oyster	8	3 925	64 97
Clams	3	0 00	11 63
Mussels	4	1 77	22 45
Abalone .	5	0 796	24 12
Cryptochiton	17	2 452	12 67
Shrimp..	6	13 07	18 65
Crab	9	2 50	30 97
Salmon..	2	4 00	8 00
Sea-lion	17	0 564	32 25
Whale	6	0 00	40 00
Total	86	0 497	4 25

Since it was impossible to get oysters in the shell, the author had to depend upon the fresh shucked oyster bought by the hundred from local markets. The amount from 100 oysters (total weight 303.6 gm., Samples O-VIII and O-IX) was found to be 6.0 mg. per kilo of tissue and liquid, and for 200 oysters bought at a different time (weight 453.67 gm.) the amount of copper was 13.7 mg. per kilo of tissue and liquid. These results are lower than those given by Rose and Bodansky (1920), namely 24 to 60 mg. per kilo, and Hiltner and Wichmann (1919), in Table IV, 6 to 30 mg. per

TABLE I—*Concluded.*

Sample No.	Animal.	Amount analyzed.	Copper in fresh material		Zinc in fresh material.		Remarks.
			Per sample.	Per kilo.	Per sample	Per kilo.	
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	
SSM-IV	Sea-lion; muscle.	100.00	Trace.	Trace.	5.00	50.0	2nd sea-lion.
SSM-V	" "	100.00	"	"	5.00	50.0	
SIS-I	" spleen.	50.00	"	"	0.50	10.0	All samples minced and sampled.
SIS-II	" "	50.00	"	"	0 60	12.0	
SIS-III	" "	100.00	"	"	1.00	10.0	Samples from 2nd sea-lion 3 weeks later.
SIS-IV	" "	102.10	"	"	1.20	11.75	
SIL-I	" liver.	50.00	"	"	2 40	48.0	
SIL-II	" "	50.00	"	"	2.40	48.0	
SIL-III	" "	122.20	"	"	5 80	48.2	
SIL-IV	" "	102.20	"	"	4 90	48.0	2nd sea-lion.
SIL-V	" "	100.20	"	"	0 50	49.0	
SIB-I	" blood.	100.00	None.	None.	0.01	1.0	
SIB-II	" "	100.00	"	"	0.01	1.0	
SIBi-I	" bile.	50.00	0.18	3.60	0.12	2.4	
WS-I	Sperm whale; liver.	100.00	None.	None.	4 00	40.0	
WS-II	" " "	100.00	"	"	4.00	40.0	Fresh samples.
WS-III	" " "	100.00	"	"	4.00	40.0	
WM-I	" " "	100.00	"	"	4.00	40.0	
WM-II	" " "	100.00	"	"	4.00	40.0	
WM-III	" " "	100.00	"	"	4.00	40.0	
	Sea water.	*cc.*					
SW-I	Sea water; near shore.	125	"	"	0 05	0 39	1 liter from near Pescadero.
SW-II	" " " "	250	"	"	0.10	0.39	
SW-III	" " 1 mile out.	500	"	"	0.01	0.002	
SW-IV	" " 1 " "	500	"	"	0.01	0.002	Per liter.

The California oyster, *Ostrea lurida,* did not seem to carry as large an amount of copper as the Eastern oyster, according to the analysis of Bodansky (1920). Samples of oysters (see Sample O-I) gave 6.6 mg. per kilo, while Sample O-VIII shows 4.3 mg. per kilo. The liquid drained from the oyster ran from 1.3 to 1.7 mg. per kilo, or for the eight samples analyzed, the average would be 3.9 mg. per kilo of tissue and liquid. This would indicate that some of the copper of oysters is to be found in the liquid.

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Clams	3	0 00	11 63
Mussels	4	1 77	22 45
Abalone . .	5	0 796	24 12
Cryptochiton	17	2 452	12 67
Shrimp..	6	13 07	18 65
Crab......	9	2 50	30 97
Salmon......	2	4 00	8 00
Sea-lion .	17	0 561	32 25
Whale .	6	0 00	40 00
Total	86	0 497	4 25

Since it was impossible to get oysters in the shell, the author had to depend upon the fresh shucked oyster bought by the hundred from local markets. The amount from 100 oysters (total weight 303.6 gm., Samples O-VIII and O-IX) was found to be 6.0 mg. per kilo of tissue and liquid, and for 200 oysters bought at a different time (weight 453.67 gm.) the amount of copper was 13.7 mg. per kilo of tissue and liquid. These results are lower than those given by Rose and Bodansky (1920), namely 24 to 60 mg. per kilo, and Hiltner and Wichmann (1919), in Table IV, 6 to 30 mg. per

kilo. The California oyster is much smaller than the Eastern variety.

The zinc content for the oysters, *Ostrea lurida*, was from 80 to 99 mg. per kilo of tissue, and from 15 to 28.44 mg. per kilo for the liquid. The amount of zinc found for the eight samples of tissue and liquid was 64.11 mg. per kilo against an average of 259.9 mg. per kilo for five samples of the Eastern oyster, analyzed by Bodansky (1920). These results obtained from the eight samples are lower than the results of Hiltner and Wichmann (1919) which range from the minimum of 146 mg. to a maximum of 779 mg. per kilo for all the samples given in their Table IV.

The clams (*Ensis americanus*) did not give a test for copper, but carried from 5.09 to 15.09 mg. of zinc per kilo. The small clams (*Venus kennicottii*) carried the least amount. The abalone showed 0.8 mg. of copper and 25 mg. of zinc per kilo, which was slightly higher than the clam. Table I shows the two varieties of starfish examined, the *Asterias orcacea* (yellow and black) which gave an average of 2.58 mg. of copper and 17.38 mg. of zinc per kilo, while the species *Asterias patria nuniaba* (red) showed an average of 1.98 mg. of copper and 19.06 mg. of zinc per kilo.

In the cryptochiton, *stelleri*, an attempt was made to find out how these metals were distributed through the animal organism. Ten cryptochiton of different sizes were cut along the side of the foot and the blood was withdrawn by a pipette. By gently folding the animal together, then straightening it out most of the blood was removed. This was mixed and sampled. The mantles were dissected from the internal shell as completely as possible. The muscle (or foot) and mantle were each minced, and each was thoroughly mixed before sampling. The internal shell was weighed directly without grinding. The results given in Tables III and IV are the average of three closely checking samples, except for the digestive organs, where only two samples were averaged.

The largest amount of copper was found in the blood, which was to be expected. The blood of the cryptochiton was colorless when drawn, but upon exposure to the air it turned a dull bluish purple color. The oxygen was carried by the copper of the hemocyanin forming the blue oxyhemocyanin. The greatest amount of zinc,

TABLE III

Distribution of Copper in the Cryptochiton (stelleri)

Tissue	Amount of tissue analyzed	Copper in sample	Copper per 100 gm	Copper per kilo
	gm	*mg*	*mg*	*mg*
Muscle	50 00	0 0533	0 1066	1 066
Mantle	50 00	0 2000	0 4000	4 000
Digestive organs	100 00	0 055	0 055	0 55
Blood	100 00	0 5166	0 5166	5 166
Internal shell	49 04	0 00	0 00	0 00

TABLE IV

Distribution of Zinc in the Cryptochiton (stelleri).

Tissue	Amount of tissue analyzed	Zinc in sample	Zinc per 100 gm	Zinc per kilo
	gm	*mg*	*mg*	*mg*
Muscle	50 00	0 0933	1 866	18 66
Mantle	50 00	2 00	4 00	40 00
Digestive organs	100 00	0 30	0 30	3 00
Blood	100 00	0 01	0 01	0 10
Internal shell	49 04	0 00	0 00	0 00

TABLE V

Comparison of Copper and Zinc Content for California and Eastern Animals

Animal	California			Eastern		
	Average No of samples	Copper	Zinc	Average No of samples	Copper	Zinc
		mg per kilo	*mg per kilo*		*mg per kilo*	*mg per kilo*
Oysters	8	3 925	64 97	5	43 85	259 88
Clams	3	0 000	11 63	1	00 00	77 00
Shrimps	6	13 07	18 65	3	13 00	17 05
Crabs	9	2 50	30 97	2	5 75	20 55
Total	26	19 495	125 22	12	62 60	374 48
Average		0 75	4 81		5 20	31 20

Table III, was found in the mantle. Just what is the function of the zinc in the animal organism has not been ascertained.

A comparison of the copper and zinc content for the California variety of oysters, crabs, shrimps, and clams, with those of the Eastern waters was made.

The results for the Eastern marine animals were taken from the analysis in Table III by Rose and Bodansky (1920), and Table

TABLE VI.

Distribution of Copper in the Tissues of the Sea-Lion and Whale.

Tissue.	Average amount of copper.			
	No of analyses.	Sea-lion.	No. of analyses.	Whale.
		mg. per kilo		*mg. per kilo*
Muscle	5	1.20	3	None.
Liver	5	Trace.	3	"
Spleen	4	"		
Blood	2	None.		
Bile	1	3.6		

TABLE VII.

Distribution of Zinc in the Tissues of the Sea-Lion and Whale.

Tissue.	Average amount of zinc.			
	No of analyses.	Sea-lion.	No of analyses	Whale.
		mg per kilo		*mg. per kilo*
Muscle	5	52.00	3	40.00
Liver	5	48 42	3	40 00
Spleen	4	10 95		
Blood	2	1.00		
Bile	1	2.40		

II by Bodansky (1920). An average was taken for all the analyses given for each animal in these tables. The copper in the Eastern marine animals is 6.93 times as great as that found for the corresponding California marine animals, while the zinc in the Eastern species is 6.48 times as great as that of the California forms.

Tables VI and VII show that mammals which live on sea food do not accumulate copper. The bile of the sea-lion (*stelleri*)

carries the greatest amount, which shows that it is thrown off by this means. Copper in small amounts has been found in the bile of land animals. Since the sea-lion has hemoglobin, instead of hemocyanin, to carry oxygen, we do not find copper in the blood. Copper was not present in samples of whale liver and muscle.

Zinc was found in varying amounts in the tissues of both the sea-lion and whale, as is shown by the table.

SUMMARY.

1. Sixteen different animals were examined and copper was present in all except the clam and whale. The average amount of copper was 0.497 mg. per kilo.
2. Copper was not present in the internal shell of the cryptochiton, the blood of the sea-lion, or the tissues of the whale. There was only a trace of copper in the mussel shells, and the liver and spleen of the sea-lion.
3. Zinc was determined and found present in all the animals. The average amount found was 4.25 mg. per kilo.
4. Zinc was not present in the internal shell of the cryptochiton, or in the mussel shells.
5. The distribution of copper and zinc in the cryptochiton was not uniform. The blood contained the greatest amount of copper and the least amount of zinc. The copper, which is in combination with the protein, unites with the oxygen to form oxyhemocyanin. The mantle carries the highest amount of zinc, but the copper content is lower than in blood.
6. The California oyster shows the greatest amount of zinc of all the samples analyzed. The zinc content of these oysters is much lower than that of the Eastern oyster.
7. Sea-lion and whale, being mammals, do not carry copper to any extent. The copper in the sea food eaten does not appear to be accumulative. The zinc was present in all tissues examined, but the amount is less than for oysters.
8. Copper is present in the lower forms of sea animals as an oxygen carrier in placé of the iron of mammals. Environment does not seem to change the sea-lion and whale in this respect.
9. The function of the zinc had not been clearly worked out, but it may be present as an aid to enzymic action in a manner not understood.

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CONPHASEOLIN.

A NEW GLOBULIN FROM THE NAVY BEAN, PHASEOLUS VULGARIS.

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A suggestion that the seed of *Phaseolus vulgaris* might contain an unknown globulin was found in certain observations of Osborne (1). This investigator isolated two proteins: a typical globulin, *phaseolin;* and a curious, atypical protein, which was named *phaselin.* Osborne's determination of the coagulation ranges of the substances in the saline extract indicated *three* proteins, however. It is improbable that any of the three precipitates consisted of albumin, for all of them formed at temperatures above 60°. Further, saline extracts of the navy bean meal used in our own experiments yielded the usual trace of albumin, coagulating between 40 and 50°, as well as the three coagula observed by Osborne.

The separation of phaseolin and phaselin was effected by fractional dialytic precipitation, phaselin being soluble in aqueous sodium chloride so dilute as to be incapable of holding in solution any appreciable trace of phaseolin, or, indeed, of any well characterized globulin. When, after prolonged dialysis, the phaselin was finally precipitated, it was found to be for the most part denatured. For this reason, and because of its unusual solubility, Osborne[1] says of it that "there is some difficulty in deciding to what class of proteids phaselin should be assigned."

Another indication which pointed in the same direction as did Osborne's observations (1) was found in some preliminary ex-

[1] Osborne (1), p. 760.

periments[2] made by A. J. Finks, of this laboratory, upon the lima bean, *Phaseolus lunatus*, and in the result obtained by the authors in an investigation of the proteins of the mung bean, *Phaseolus aureus* (4). In both cases a readily denaturable α-globulin[3] of high sulfur content was isolated, together with a much larger quantity of a β-globulin, closely resembling phaseolin in precipitability by ammonium sulfate, in other physical properties, and in chemical composition. It seemed a reasonable supposition, therefore, that the third protein indicated in Osborne's experiments on *Phaseolus vulgaris* would prove to be an α-globulin of the type previously found in the seed of *Phaseolus lunatus* and *Phaseolus aureus;* and that probably it would be most readily separable from phaseolin and phaselin by fractionation with ammonium sulfate and subsequent dialysis of the principal fractions.

Experiment confirmed this conjecture. When an aqueous 2 per cent sodium chloride extract of navy bean meal was treated with ammonium sulfate, precipitation began at about 0.18 of saturation, and when the concentration of the precipitant reached 0.25 of saturation a flocculent precipitate settled upon allowing the solution to stand for a few minutes. Purified phaseolin (1) does not begin to precipitate at a concentration of ammonium sulfate less than 0.64 of saturation, and requires 0.88 of saturation for complete precipitation. In the mixed extract the precipitation of the second main fraction begins at a slightly lower concentration of the salt, but even in this case no visible separation takes place below 0.57 of saturation.

The new protein, or *conphaseolin*, as we have called it, was found to have an average sulfur content of 1.36 per cent; and,

[2] Since completed and published (2). After the first preparation of conphaseolin had been made and analyzed, the work on the navy bean was laid aside temporarily to give place to a more important investigation. Meanwhile, a similar study of the proteins of the adsuki bean, *Phaseolus angularis* (3), has shown that that seed also, yields an α-globulin similar in all respects to those of the lima, mung, and navy beans.

[3] By α-globulin is meant a globulin precipitable by ammonium sulfate at a relatively low concentration, as the first of two principal globulin fractions of clearly different chemical identity. The second globulin in such cases is, of course, designated the β-globulin. The same terminology is used throughout this paper except where names for the proteins have been constructed from the botanical name of their source.

in general, its chemical and physical properties were strikingly different from those of either phaseolin or phaselin, and very similar to those of the α-globulins found previously in the lima (2) and mung (4) beans; and, subsequently, in the adsuki bean, *Phaseolus angularis* (3).

EXPERIMENTAL.

Coagulation Points of the Proteins in the Saline Extract.—25 cc. of a 2 per cent aqueous sodium chloride extract of navy bean meal were treated with 2 or 3 drops of 2 per cent acetic acid, and the test-tube containing them, set up in a water bath (beaker), was heated at the rate of about 0.5° per minute. A dark background was placed behind the apparatus, and a strong light, passing over the top of the background and diagonally downward through the test-tube, made distinctly visible the slightest turbidity in the solution under examination. Under these conditions four coagulations were observed. The first appeared as a cloud at 40°, gradually increasing in density, and assuming a granular quality between 49 and 50°. Heated for an hour between 49.9 and 50.5° it clotted and settled, leaving the solution quite clear. The filtrate from this precipitate was returned to the tube and again heated. A second coagulum was obtained between 62 and 74.6°; a third from 78.5 to 83°; and from 86 to 100°, a fourth and rather heavy coagulum was formed.

The small quantity of the first precipitate, together with the low temperature at which it separated, suggested an albumin. This view was later confirmed when it was found that the saline extracts yielded, after prolonged dialysis, a solution free from chlorides and containing but one coagulable fraction, a trace only, having the coagulation range 42 to 50° under the conditions above described.

Fractionation with Ammonium Sulfate.—For the preliminary test 180 cc. of an aqueous 2 per cent sodium chloride extract of ground navy beans were used. The ammonium sulfate was added in saturated solution from a reservoir burette. The results can most easily be presented in tabular form (Table I). They may be briefly summarized as follows: (*a*) A rather small first fraction is precipitated at about 0.25 of saturation with the precipitant. (*b*) An intermediate fraction is precipitated between

0.38 and 0.52 of saturation. After filtering off this fraction, washing it with 0.52 saturated ammonium sulfate containing 2 per cent of sodium chloride, and redissolving it in aqueous 2 per cent sodium chloride, a coagulation test showed it to be a mixture of probably as many as three proteins of different coagulation

TABLE I.

Preliminary Fractionation with Ammonium Sulfate.

Volume of saturated ammonium sulfate solution added.	Total volume, solution + precipitant.	Fraction of saturation with precipitant.	Effect noted.
cc.	*cc.*	*per cent*	
0	180	0.0	
20	200	10.0	Slightly increased opalescence.
20	220	18.2	Distinct turbidity.
10	230	21.8	Increased turbidity, no flocculation on standing.
10	240	25.0	Flocculent precipitate, settled in a few minutes.
Fraction I filtered off; experiment continued with 150 cc. of filtrate.			
25	175	35.7	No result.
5	180	37.5	Opalescence.
15	195	42.3	Turbidity.
20	215	52.3	Precipitate became flocculent and settled; quantity very small.
Fraction II filtered off; experiment continued with 160 cc. of filtrate.			
19	179	57.4	Thin, whitish cloud.
			*
31	210	63.7	Heavy, white precipitate, almost immediately flocculent.

* Intermediate stages in precipitation of Fraction III entirely similar to those detailed in connection with Fractions I and II, above.

ranges. (*c*) A β-fraction, evidently *phaseolin*, since its ammonium sulfate precipitation limits are practically identical with those reported by Osborne (1) for dialyzed and redissolved phaseolin, begins to appear when the concentration of the precipitant is brought to 0.57 of saturation; and as much of it as can be precipitated at all by ammonium sulfate in neutral solution is thrown out

by a little less than 0.80 of saturation. (*d*) The filtrate from the phaseolin precipitate contains no protein coagulable by heat in the presence of very dilute acetic acid.

Isolation of Conphaseolin; and Preparation of Phaseolin by the Ammonium Sulfate Method.

The beans were ground to a fine flour and extracted in lots of from 2 to 4 kilos with 5 volumes of aqueous 2 per cent sodium chloride.

Bean meals, containing protein partially dissolved by saline extractants, frequently show a tendency to settle in a thick, more or less gelatinous mass at the bottom of the extraction vessel—a condition far from favorable, of course, to rapid or complete solution of the proteins. To prevent this an electrically driven stirrer was used. The apparatus consisted essentially of an upward acting propeller, operating in the lower end of a stationary vertical jacket. The jacket was provided with radial vanes so that the liquid within it would not revolve with the motion of the propeller, but was forced by its upward thrust to pass up through the cylinder and over its top into the main bulk of the mixture again. When operated with the propeller about an inch above the bottom of the bucket in which the extraction was being made, the device drew up the meal from the bottom as fast as it settled, and was very effective in keeping it evenly distributed throughout the solvent. Under these conditions an extraction period of 2 hours gave satisfactory results.

The thin mush of meal and extract was prepared for pressing in the usual way, by the addition of filter paper scrap sufficient to produce a pulp that would prevent the bursting of the cloth press bags and retain most of the suspended matter. The turbid expressed liquid, about 85 per cent of the original volume of solvent, was filtered clear by suction through thick mats of filter paper pulp. This pulp was made in 2 per cent sodium chloride. If distilled water alone is used, globulins are precipitated in the filter, and the yield of readily denaturable globulins of the type of conphaseolin may be considerably diminished.

Isolation of Conphaseolin.—In the similar case of the α-globulin of the mung bean (4), it was found advantageous to use a concen-

tration of ammonium sulfate 0.05 of saturation higher than the apparent upper limit as indicated by the preliminary test, since the precipitate formed under these conditions settled more rapidly and was more easily filtered off and washed than that formed when no excess of the precipitant was present. The same procedure was therefore applied for the separation of the first fraction from the navy bean extract. The extract was treated with finely ground ammonium sulfate in small portions, each being completely dissolved before another was added, until the concentration of the salt reached 0.30 of saturation, an excess of 0.05, with a margin of 0.08 of saturation between this point and that at which, in the preliminary experiment, the middle fraction began to appear.

The remaining steps in the isolation of conphaseolin, together with the properties of the protein observed in these operations, were exactly the same as with the analogous α-globulin from the mung bean (4).

Conphaseolin is precipitated by dialysis in spheroids so minute as to be scarcely distinguishable under a magnification of 500 diameters. It is for the most part denatured during the dialysis, but that which remains soluble in saline solutions has the same precipitation limits with ammonium sulfate as before its separation from the other proteins of the original extract. The dried product is a pale greenish gray, granular powder, in quantity amounting to about 0.35 to 0.4 per cent of the dry weight of the ground beans. It is entirely insoluble in neutral solvents, and soluble only with some difficulty in 0.1 N acids and alkalies.

In elementary composition conphaseolin differs markedly from both phaseolin and phaselin. A comparative summary of the analyses of the three proteins will be found in Table II.

The nitrogen distribution of conphaseolin is given in Table IV, and the percentages of the hexone bases in Table V.

The Middle Fraction.—This was precipitated by raising the concentration of ammonium sulfate in the filtrate from the conphaseolin to 0.5 of saturation. As in the preliminary experiment, a coagulation-range test indicated that it was a mixture, apparently of all the proteins present in the original extract. The discarding of a considerable intermediate fraction is, of course, wasteful of material, but it seems the only means at present available by which reasonably pure fractions may be obtained. The first

case, that of the Georgia velvet bean (5), in which we made an ammonium sulfate fractionation in this way showed that it was possible thus to secure a β-fraction free from chemically detectable traces of the α-fraction. The β-globulin referred to contained either no tryptophane at all or so little that it gave no response in the sensitive test of Hopkins and Cole, while the α-globulin gave a strongly positive test. On the basis of present information, however, we consider it highly improbable that one fractionation of this sort, even when a middle fraction of generous proportions is removed, yields a β-fraction free from all contamination with lower fractions. Probably a higher degree of purity in both fractions, where two only are known to be present, might be

TABLE II.

Summary of Elementary Composition of Conphaseolin, Phaseolin, and Phaselin.

	Sulfur.	Nitrogen.	Carbon.	Hydrogen.	Oxygen.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Conphaseolin..................	1.36	15.73	53.81	6.86	22.24
Phaseolin*.....................	0.33	16.13	52.56	6.81	24.17
" †.....................	0.36	15.83	52.66	6.93	24.22
Phaselin‡......................	0.49	14.65	51.60	7.02	26.24

* Ammonium sulfate method; our preparations.

† Osborne and Harris's preparations; fractional dialytic precipitation method.

‡ Figures of Osborne (1).

obtained by applying the principle of repeated and multiple fractionation used in fractional distillation and crystallization. Particularly in cases where the precipitation limits are so vague in the first attempt at fractionation as to suggest a single protein of wide range of precipitability, such a further extension of the fractional precipitation method might prove useful. Whether or not the removal of a middle fraction yields, in a single fractionation, absolutely pure β-fractions can probably be determined at present only by the anaphylactic reaction.

Preparation of Phaseolin by the Ammonium Sulfate Method.—The filtrate from the middle fraction was saturated with ammonium sulfate. Phaseolin was precipitated in the preliminary experiment by a concentration just under 0.80 of saturation. As

no further precipitate was produced on saturating the filtrate from the phaseolin, however, no impurity could be introduced into the preparation by saturating with the precipitant, and this was much the more convenient procedure. The heavy white precipitate was filtered off, redissolved with distilled water (the

TABLE III.

*Elementary Composition of Conphaseolin.**

	Preparation 1.			Preparation 2.		
	I	II	Average.	I	II	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon	54.21		54.21	54.16		54.16
Hydrogen	6.92		6.92	6.91		6.91
Nitrogen	15.78	15.67	15.73	15.67	15.59	15.63
Sulfur	1.28		1.28	1.37		1.37
Oxygen†	21.81		21.86	21.89		21.93
Ash			2.24			

	Preparation 3.			Preparation 4.			Preparation 5.
	I	II	Average.	I	II	Average.	I
Carbon	53.29		53.29	54.03	54.96	54.00	53.37
Hydrogen	6.91		6.91	6.82	6.87	6.85	6.80
Nitrogen	15.86		15.86	15.69	15.82	15.76	15.88
Sulfur	1.33	1.29	1.31	1.49		1.49	1.45
Oxygen†	22.61		22.63	21.95		21.88	22.50
Ash			0.61				0.39

* On the basis of moisture- and ash-free protein. The yields of conphaseolin were so small that complete duplicate analyses were in most cases impossible.

Most of these analyses, except that of the first preparation, were done by Mr. Phillips and Mr. Gersdorff of the Protein Investigation Laboratory.

† By difference.

ammonium sulfate retained by the precipitate being sufficient in this case to furnish the saline medium necessary to the solution of the globulin), and precipitated by dialysis. The precipitate consisted for the most part of microscopic, vitreous spheroids, although a few hexagonal plates were observed in one of the preparations. Osborne has obtained this protein in crystalline

form. But inasmuch as the ammonium sulfate precipitation enabled us to secure preparations evidently of a fair degree of purity, their sulfur content being very uniform and noticeably

TABLE IV.

*Distribution of Nitrogen in Conphaseolin as Determined by Van Slyke's Method.**

Sample I, moisture- and ash-free, 2.7548 gm. protein, 0.4311 gm. nitrogen.†

Sample II, moisture- and ash-free, 2.7542 gm. protein, 0.4300 gm. nitrogen.

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0320	0.0316	7.42	7.35	7.39
Humin N adsorbed by lime	0 0126	0.0117	2.92	2.72	2.82
" " in amyl alcohol extract	0 0008	0.0008	0.19	0.19	0.19
Cystine N	0 0038	0.0038	0.88	0.88	0.88
Arginine N	0.0611	0.0608	14.17	14.14	14.15
Histidine N	0.0059	0 0068	1.37	1.58	1.47
Lysine N	0.0565	0.0566	13.11	13.16	13.14
Amino N, filtrate	0 2477	0.2477	57.46	57.60	57.53
Non-amino N, filtrate	0.0113	0.0109	2.62	2.54	2.58
Total N recovered	0.4317	0.4307	100.14	100.16	100.15

* Nitrogen figures corrected for the solubilities of the phosphotungstates of the basic amino-acids.

† Nitrogen content of the preparation analyzed, 15.65 per cent.

TABLE V.

The Hexone Bases and Cystine in Conphaseolin as Determined by Van Slyke's Method.

Amino-acid	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	1.18	1.18	1.18
Arginine	6.88	6.85	6.87
Histidine	0.79	0.91	0.85
Lysine	10.68	10.70	10.69

lower than that of the earlier preparations, it seemed unnecessary to make use of crystallization as a means of purification. The elementary composition of four preparations made in this way,

together with an average compared with the average of Osborne's analyses of the preparations made by dialytic fractionation, is given in Tables III and VI, the analyses by Van Slyke's (6) method, in Tables VII and VIII.

TABLE VI.

Elementary Composition of Phaseolin.

*	Preparation 1.			Preparation 2.		
	I	II	Aver-age	I	II	Aver-age.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon	52.55	52.47	52.51	52.53	52.58	52.56
Hydrogen	6.90	6.65	6.78	6.87	6.91	6.89
Nitrogen	16.39	16.30	16.35	15.95	15.98	15.97
Sulfur	0.332		0.332	0.329		0.329
Oxygen†	23.83	23.92	23.88	24.42	24.20	24.31
Ash			0.43			0.67
	Preparation 3.			Preparation 4.		
	I	II	Aver-age.	I	II	Aver-age.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon	52.62	52.52	52.57	52.50	52.53	52.52
Hydrogen	6.77		6.77	6.81	6.84	6.83
Nitrogen	15.85	15.98	15.92	16.00	16.07	16.04
Sulfur	0.321		0.321	0.318		0.318
Oxygen†	24.44	24.41	24 42	24.37	24.24	24.31
Ash			0.51			0.58

* Basis of moisture- and ash-free protein.
† By difference.

The slightly lower sulfur content of the preparations made by the ammonium sulfate method suggests that they are more nearly free from conphaseolin than were those made by the dialytic fractionation.

Our preparations were entirely soluble in 2 per cent sodium chloride after dialysis, and were precipitable by ammonium sulfate within limits exactly the same as those given by Osborne.

TABLE VII.

*Distribution of Nitrogen in Phaseolin as Determined by Van Slyke's Method.**

Sample I, moisture- and ash-free, 2.7204 gm. protein, 0.4436 gm. nitrogen.†
Sample II, moisture- and ash-free, 2.7200 gm. protein, 0.4435 gm. nitrogen.

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0489	0.0482	11.03	10.87	10.95
Humin N	0.0031	0.0032	0.70	0.72	0.71
Cystine N	0.0037	0.0037	0.83	0.83	0.83
Arginine N	0.0556	0.0559	12.53	12.60	12.57
Histidine N	0.0189	0.0159	4.26	3.59	3.93
Lysine N	0.0487	0.0497	10.98	11.21	11.10
Amino N, filtrate	0.2662	0 2643	60.01	59.60	59.81
Non-amino N, filtrate	0.0001	0 0015	0.02	0.34	0.18
Total N recovered	0.4452	0.4424	100.36	99.76	100 08

* Nitrogen figures corrected for the solubilities of the phosphotungstates of the basic amino-acids.

† Nitrogen content of the ash- and moisture-free protein, 16.30 per cent.

TABLE VIII.

The Basic Amino-Acids in Phaseolin as Determined by Van Slyke's Method.*

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	1.16	1.16	1.16
Arginine	6.34	6.38	6.36
Histidine	2.56	2.15	2.36
Lysine	9.32	9.51	9.42

* The basic amino-acids in phaseolin as prepared by direct dialysis, without fractionation with ammonium sulfate, have been determined by Finks and Johns (Finks, A. J., and Johns, C. O., *J. Biol. Chem.*, 1920, xli, 375).

SUMMARY.

Fractionation of a 2 per cent aqueous sodium chloride extract of the navy bean, *Phaseolus vulgaris*, with ammonium sulfate as the precipitant separated the protein content of the extract

into two principal fractions. One of these proved to be identical with the phaseolin previously described by Osborne (1), but the other, the α-fraction in ammonium sulfate precipitation, proved to be a hitherto unknown globulin, having a chemical composition which differentiated it sharply from both of the proteins (*phaseolin* and *phaselin*) isolated by Osborne. The name *conphaseolin* has been assigned to the new substance. It is distinguished from phaseolin and phaselin most conspicuously by its very much higher sulfur content. In nitrogen and in carbon content also, however, as well as in certain of its physical properties, it is notably different from either of the two proteins with which it is associated.

Conphaseolin is, in fact, an α-globulin of the same type as those previously found in the lima and mung beans (*Phaseolus lunatus* and *aureus*), and subsequently in the adsuki bean (*Phaseolus angularis*). It has the typical general properties of the group. It is precipitable by ammonium sulfate at a relatively low concentration, is readily denaturable, and possesses a high carbon content and a very high sulfur content.

The analysis of conphaseolin by Van Slyke's method indicates an extraordinarily large percentage of lysine. The figure (10.69 per cent) is in fact the highest for this amino-acid which has thus far been found for any vegetable protein.

Phaseolin prepared by the ammonium sulfate fractionation method has a slightly lower sulfur content than had the preparations of Osborne and Harris, isolated by fractional dialytic precipitation. This may be due to the more complete separation of traces of conphaseolin from the phaseolin preparations by the ammonium sulfate method.

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THE RATE OF UREA EXCRETION.

V. THE EFFECT OF CHANGES IN BLOOD UREA CONCENTRATION ON THE RATE OF UREA EXCRETION.*

BY T. ADDIS AND D. R. DRURY.

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(Received for publication, December 8, 1922.)

There are many other factors than blood urea concentration which may influence the rate of urea excretion. It is only under conditions in which some of these other factors are eliminated and the remainder happen to counterbalance one another that it can be shown that in the same individual the rate varies directly with the blood urea concentration. The measurements given in Tables I and II, and charted in Figs. 1 and 2, show that under the conditions selected the rate of urea excretion in the two subjects we examined varies only with the blood urea concentration and in such a way that with every increase in blood urea concentration there is a proportionate increase in the rate. These conditions were as follows. At an early hour in the morning, usually about 6 a.m., the subject drank about 1,000 cc. of water in which a variable amount of urea had been dissolved. No food was taken. Every hour thereafter about 500 cc. of water were drunk. No measurements were made until at least 3 hours after the administration of urea. The urine was collected at hourly intervals and blood was drawn at the middle of each period of urine collection.

When no special precautions are taken to eliminate the action of other factors than blood urea concentration wide variations

* This work was aided by a grant from the Committee on Scientific Research of the American Medical Association.

A preliminary report of the results in this paper was presented to the Society for Experimental Biology and Medicine (Addis, T., and Drury, D. R., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 38).

TABLE I.

Effect of Variations in Blood Urea Concentration on the Ratio of Urea Excretion.

Special conditions which were constant except for variation in the amounts of urea taken.
Subject: Add.

Blood urea.	Urine urea.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.
mg. per 100 cc.	*mg. per hr.*	
17.7	750	42.4
19.3	978	50.7
20.2	894	44.3
22.5	1,107	49.2
34.5	1,740	50.4
39.0	1,987	50.9
40.5	1,764	43.6
42.0	2,010	47.9
45.0	1,940	43.1
46.5	2,370	51.0
47.8	2,110	44.2
49.5	2,070	41.8
52.8	2,300	43.6
54.0	2,340	43.3
56.6	2,560	45.2
57.2	2,570	44.9
57.3	2,780	48.5
61.2	3,120	51.0
61.8	2,820	45.6
62.0	3,050	49.2
65.9	2,840	43.1
66.8	2,870	43.0
69.4	3,570	51.4
74.9	3,300	44.1
80 4	3,400	42.3
86.5	3,930	45.4
93.5	4,220	45.1

Average.. 46.1
Standard deviation...................................$\pm$3.16
Variability...$\pm$6.85 per cent

from any direct relationship are observed. Fig. 3 shows rates determined on the subject Add. under the ordinary conditions of every-day life with the exception that variable amounts of

TABLE II.

Effect of Variations in Blood Urea Concentration on the Rate of Urea Excretion.

Special conditions which were constant except for variation in the amounts of urea taken.

Subject: Dru.

Blood urea.	Urine urea.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.
mg. per 100 cc.	*mg. per hr.*	
20.2	1,180	58.4
21.0	1,130	53.8
21.0	1,160	55.2
21.7	1,220	56.2
23.2	1,320	56.9
24.0	1,320	55.0
25.5	1,430	56.1
27.7	1,480	53.4
29.2	1,460	50.0
40.1	2,020	50.4
42.1	2,260	53.7
44.2	2,700	61.1
45.1	2,440	54.1
47.5	2,580	54.3
49.7	2,910	58.6
52.3	2,770	53.0
53.6	3,100	57.8
54.7	3,200	58.5
54.8	2,980	54.4
57.0	3,490	61.2
57.4	3,310	57.7
59.9	3,220	53.8
61.0	3,560	58.4
66.6	3,730	56.0
72.8	4,280	58.8
78.9	4,490	56.9
85.0	4,960	58.4
88.5	5,220	59.0
91.2	5,300	58.1
97.5	5,620	57.7
108.0	6,100	56.5

Average.. 56.2
Standard deviation.............................. ±2.68
Variability..................................... ±4.76 per cent

urea and water were sometimes taken. This is the same subject whose rates under appropriate conditions showed the direct relation demonstrated in Fig. 1.

The conclusion that the rate of urea excretion tends in a general way and on the average to vary directly with the blood urea concentration might be drawn even from Fig. 3, and such a deduction has indeed already been drawn from somewhat similar data by

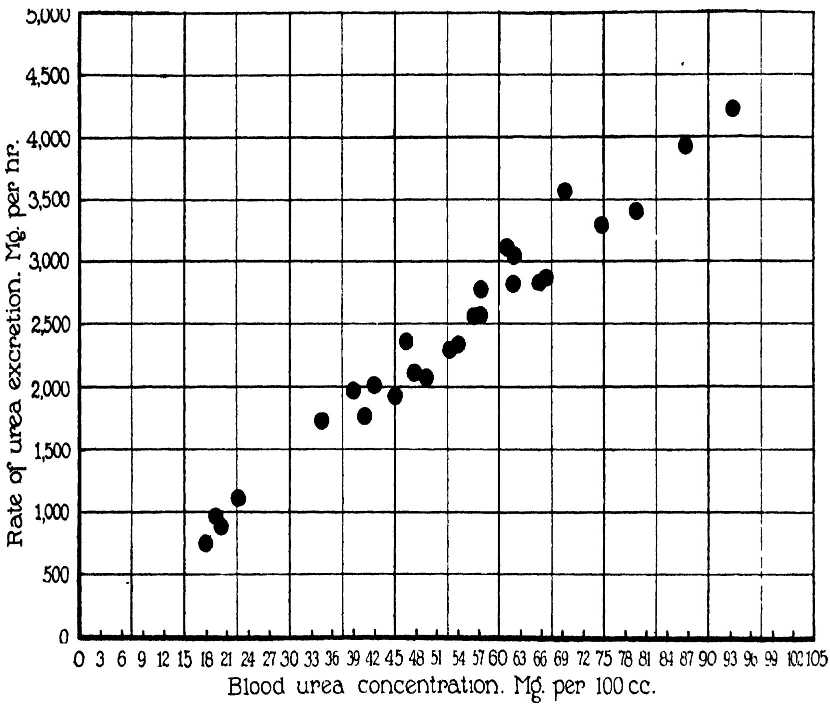

FIG. 1. The effect of changes in blood urea concentration on the rate of urea excretion of the subject Add. under standard conditions.

Marshall and Davis,[1] Addis and Watanabe,[2] and Austin, Stillman, and Van Slyke.[3] But the knowledge of the existence of this general tendency towards a direct relationship is not enough

[1] Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.
[2] Addis, T., and Watanabe, C. K., *J. Biol. Chem.*, 1917, xxix, 391.
[3] Austin, J. H., Stillman, E., and Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlvi, 91.

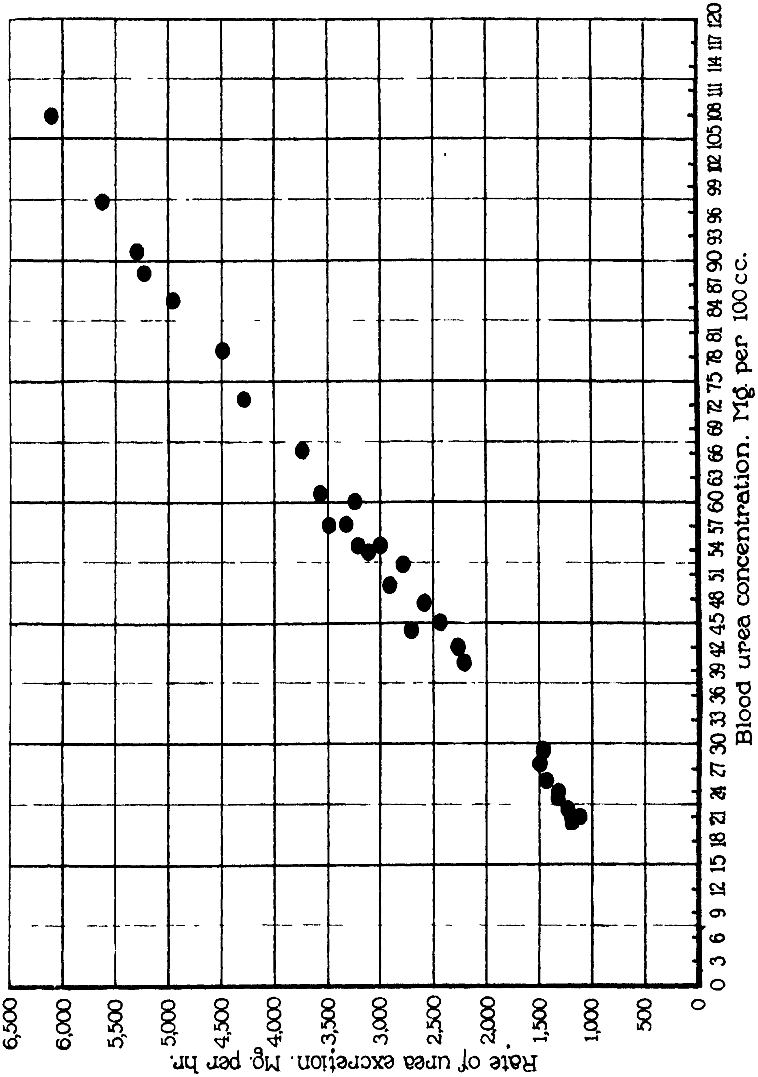

FIG. 2. The effect of changes in blood urea concentration on the rate of urea excretion of the subject Dru. under standard conditions.

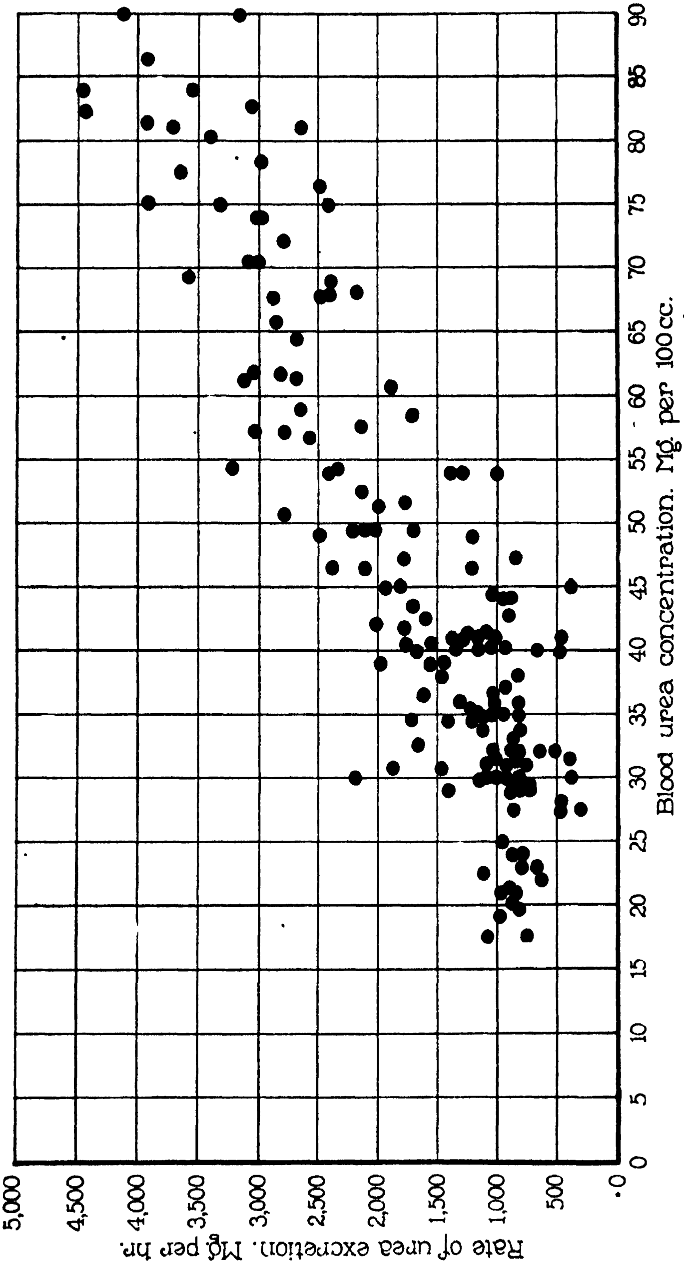

Fig. 3. The effect of changes in blood urea concentration on the rate of urea excretion of the subject Add. under the ordinary conditions of every-day life.

for the purposes of an investigation into the various other factors than blood urea concentration which may influence the rate. For experimental purposes a knowledge of the particular conditions under which the ratio: $\frac{\text{urea in 1 hour's urine}}{\text{urea in 100 cc. of blood}}$ becomes within narrow limits a constant for any one individual is important, because the effect of any given factor can be measured by the nature and the degree of change it produces in the ratio. These ratios are given in Tables I and II. For Add. the average ratio is 46.1 with a standard deviation of ± 3.16 and a variability of ± 6.85 per cent. For Dru. the average is 56.2 with a standard deviation of ± 2.68 and a variability of ± 4.76 per cent.

CONCLUSION.

Under certain special conditions the rate of urea excretion becomes directly proportional to the blood urea concentration, so that in any one individual the ratio: $\frac{\text{urea in 1 hour's urine}}{\text{urea in 100 cc. of blood}}$ is a constant with only narrow limits of variation, over a wide range of blood urea concentration.

THE RATE OF UREA EXCRETION.

VI. THE EFFECT OF VERY HIGH BLOOD UREA CONCENTRATIONS ON THE RATE OF UREA EXCRETION.*

By D. R. DRURY.

(*From the Department of Medicine of Stanford University Medical School, San Francisco.*)

(Received for publication, December 8, 1922.)

In the preceding paper it has been shown that under certain special conditions the rate of urea excretion increases in direct proportion with every increase in blood urea concentration up to a concentration of about 100 mg. per 100 cc. The experiments given in this paper were carried through in order to determine the effect on the rate of still greater increases in blood urea concentration.

Male rabbits of from 2.2 to 2.5 kilos in weight were used. The anesthetic was 150 mg. of chlorotone dissolved in alcohol and given by stomach tube. An hour after the administration of the chlorotone the injection of varying concentrations of urea into the ear vein was commenced. A Woodyatt pump was used and the temperature of the fluid as well as the rate of injection was kept constant. After the injection had continued for an hour and diuresis was thoroughly established, the bladder was washed out and when the last wash water had been recovered the time was taken for the commencement of the first urine collection. The catheter was left in position and an hour later the bladder was washed out and the collection of urine completed. Three such hourly collections were usually made in sequence. At the middle of each period of urine collection blood was obtained from the ear vein. Three experiments were usually carried through on each animal, allowing a week or more between each. During one experiment only 0.9 per cent sodium chloride solution was injected;

* This work was aided by a grant from the Committee on Scientific Research of the American Medical Association.

TABLE I.

Blood urea.	Urine urea.	Ratio: $\frac{\text{urine urea}}{\text{blood urea}}$.	Urine volume.	Rate of injection.	Urea concentration injected in 0.9 per cent NaCl.
		Rabbit 1.			
mg. per 100 cc.	*mg. per hr.*		*cc. per hr.*	*cc. per hr.*	*gm. per 100 cc.*
27	97	3.59	16	60	0
27	91	3.37	8	60	0
149	533	3.58	53	60	2
166	462	2.82	27	60	2
174	541	3.11	39	60	2
377	1,470	3.91	68	100	2.5
482	2,070	4.44	116	100	2.5
755	4,020	5.32	265	180	5.0
1,004*	3,610	3.60	162	180	5.0
1,305*	4,030	3.09	156	180	5.0
		Rabbit 2.			
19	62	3.26	11	60	0
19	79	4.19	23	60	0
19	80	4.22	17	60	0
150	628	4.19	46	60	2
157	670	4.79	39	60	2
166	798	4.69	59	60	2
177	831	4.27	51	60	2
292	1,070	3.66	58	100	3
327	1,330	4.06	57	100	3
346	1,560	4.50	67	100	3
		Rabbit 3.			
166	370	2.23	19	60	2
186	590	3.17	27	60	2
204	580	2.84	42	60	2
234	788	3.37	43	60	2
355	1,180	3.31	73	200	3
414	1,570	3.80	102	200	3
445	1,850	4.16	124	200	3
		Rabbit 4.			
33	116	3.55	12	60	0
33	137	4.18	17	60	0
152	485	3.19	18	60	2
169	662	3.92	29	60	2
188	774	4.13	27	60	2
470	1,470	3.12	94	200	3
528	2,020	3.82	131	200	3
567	1,860	3.29	139	200	3

* Convulsions.

TABLE I—*Concluded*

Blood urea	Urine urea	Ratio $\frac{\text{urine urea}}{\text{blood urea}}$	Urine volume	Rate of injection	Urea concentration injected in 0 9 per cent NaCl
			Rabbit 5.		
mg per 100 cc	*mg per hr*		*cc per hr*	*cc per hr*	*gm per 100 cc.*
17	64	3 70	22	60	0
17	64	3 70	11	60	0
166	559	3 37	24	60	2
197	709	3 60	44	60	2
452	2,320	5 13	215	200	3
572	2,590	4 48	190	200	3
674	2,880	4 27	171	200	3

during another, 2 per cent urea in sodium chloride solution; and during another, either 3 per cent urea was given or the rate of injection of 2 per cent urea was increased. The order in which these injections were made had no apparent effect on the results. With the above procedure it was possible to measure the rate at physiological levels of blood urea concentration, at concentrations between 100 and 300 mg. per 100 cc. and at concentrations between 300 and 800 mg. per 100 cc. The measurements are given in Table I and the rates for each of the five animals used are plotted in Fig. 1 against the observed blood urea concentrations.

Fig. 1 shows that in general the rate is directly proportional to the blood concentration even when the concentration is forced to 780 mg. per 100 cc. There are, it is true, irregularities in the curves but there is no level of blood urea concentration beyond which the rate ceases to rise as the concentration goes still higher.

In experiments in which urea was given by stomach tube to rabbits, Addis, Shevky, and Bevier[1] found that the average rate of excretion of a large group of animals ceases to increase in direct proportion with the blood urea concentration when the concentration exceeded 225 mg. per 100 cc. From this observation, though with some hesitation, they concluded that at levels above 225 mg. per 100 cc. the urea-secreting tissue of the kidney was quantitatively insufficient for the task of handling the very large

[1] Addis, T., Shevky, A. E., and Bevier, G., *Am. J. Physiol.*, 1918, xlvi, 11.

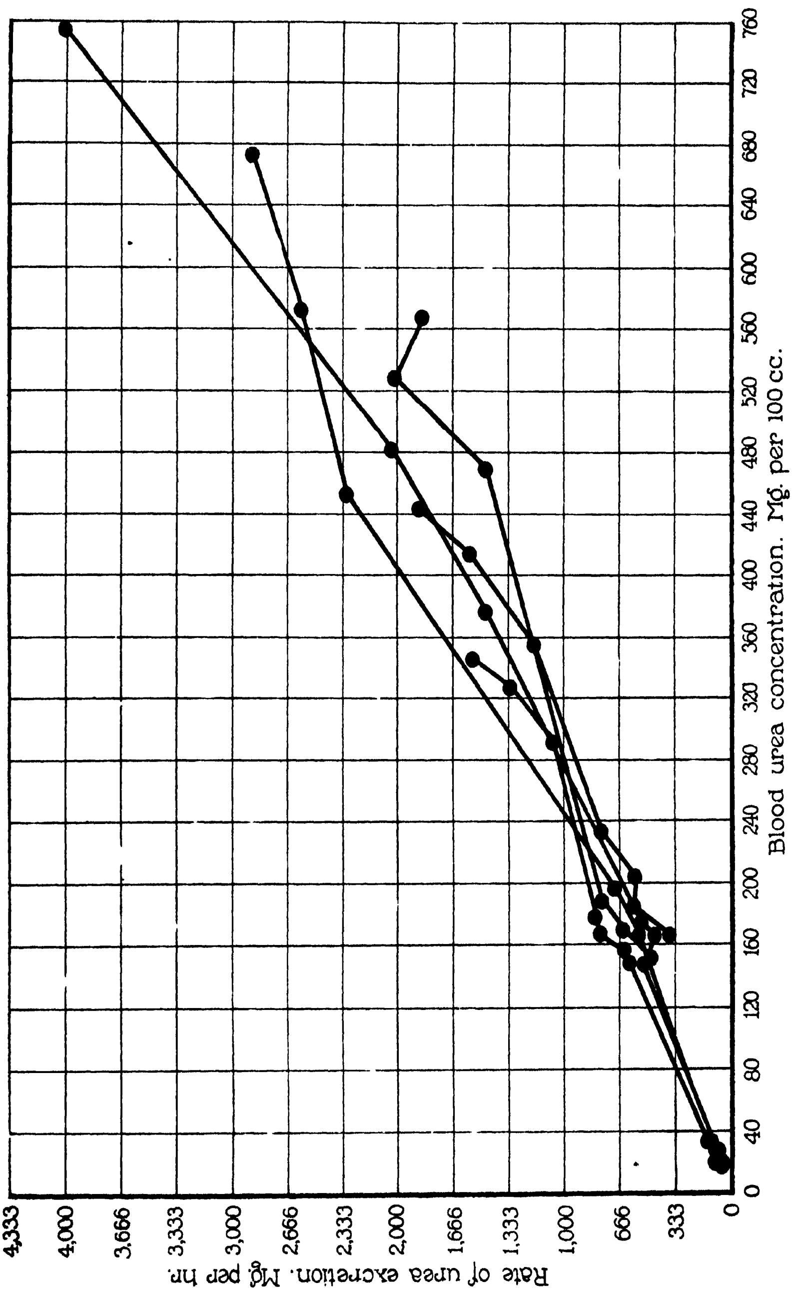

FIG. 1. The rate of urea excretion at very high blood urea concentrations.

amounts of urea which would have to pass through it if a direct relation between the rate and the concentration were to be maintained. The experiments given in this paper show that that conclusion was erroneous. The failure of many of their animals to respond adequately when the blood urea concentration became very high was probably due to minor and unrecognized grades of ammonia poisoning. Rabbits not infrequently die from ammonia poisoning when the large amounts they were using in these experiments are given by stomach tube.[2] There were eight deaths from ammonia poisoning among 131 of their animals to whom 5 gm. of urea were given in this manner. When 10 gm. were given the percentage of fatal cases was much larger and only two of seven rabbits who received 15 gm. survived. It was, of course, among those rabbits who were given these large quantities that the failure of the rate to keep pace with very high blood urea concentrations was observed. The cause of the condition is almost certainly the failure to absorb all the urea from the stomach and small intestine so that part of it reaches the large intestine, is decomposed there by urease-containing organisms, and the resulting ammonia taken up by the blood stream. When the urea is given intravenously as in our experiments much higher blood urea concentrations can be reached without danger of ammonia poisoning, and under these conditions the evidence is clear that at all blood urea concentrations consonant with life the rate remains directly proportional to the concentration. It is, indeed, not possible to reach any absolute limit to the urea-excreting capacity of the kidney because when very large amounts of urea are injected general toxic manifestations appear which indirectly may lead to a decrease in renal activity. Thus Rabbit 1 went into convulsions when the blood urea concentration was forced to over 1,000 mg. per 100 cc. and died soon after although the kidneys were still excreting urea at an extraordinary rate.

It will be noted that we obtained some very high rates of urea excretion. In this respect the results confirm and extend the observations made by Herter[3] many years ago. The greatest rate seen by Herter in the rabbit after intravenous injection of urea was 482 mg. of urea per hour per kilo of body weight. In

[2] Barnett, G. D., and Addis, T., *J. Biol. Chem.*, 1917, xxx, 41.

[3] Herter, C. A., *Johns Hopkins Hosp. Rep.*, 1900, ix, 69.

one of our rabbits 4,020 mg. of urea per hour were excreted, or 1,785 mg. per hour per kilo of body weight. This is equivalent to the excretion by a man of 70 kilos of 3,000 gm. of urea in 24 hours, an amount about 200 times greater than that which is ordinarily eliminated.

CONCLUSION.

The rate of urea excretion continues to increase in direct proportion to increase in blood urea concentration even when the concentration rises to over 700 mg. per 100 cc.

THE SUPPLEMENTARY PROTEIN VALUE OF PEANUT FLOUR.

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(From the Department of Physiological Chemistry, Teachers College, Columbia University, New York.)

(Received for publication, December 7, 1922.)

In 1920 Johns and Finks[1] reported a series of experiments whose results suggested the desirability of utilizing peanut flour as a supplement to wheat flour. In this article they state: "Bread made with a mixture of 25 parts of peanut flour and 75 parts of wheat flour [74 per cent extraction] furnished adequate proteins and water-soluble vitamine for normal growth." These authors had previously shown[2] that the principal proteins of the peanut are two globulins to which they have given the names of arachin and conarachin and that these proteins are particularly rich in the basic nitrogen that is so lacking in wheat flour proteins. Their findings in this respect are given in Table I.

The value of peanut flour as a nutritive product was also demonstrated in 1918 by Daniels and Loughlin.[3] These investigators reported that a purified food mixture, yielding 18 per cent protein and composed of 67.5 gm. of peanut meal plus 10 gm. of lard, 5 gm. of butter fat, 11.4 gm. of starch, and 5.09 gm. of suitable inorganic materials, was in every way adequate for normal growth and reproduction of rats.

In 1919, and again in 1921, McCollum, Simmonds, and Parsons[4] called attention to a method of comparing the supplementary protein value of foodstuffs. In the earlier paper they maintained that it was possible to secure normal growth with an otherwise adequate diet if the protein content was as low as 9 per cent provided that the quality of the protein was high. Making use of this method the later papers detail an extended application of the method to foodstuffs. The following quotation from the 1921 papers summarizes their findings in part: ". . . kidney, liver, and muscle proteins have much greater values as supplements to the

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[1] Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, 1920, xlii, 569.

[2] Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1916–17, xxviii, 77; 1917, xxx, 33; 1918, xxxvi, 491.

[3] Daniels, A. L., and Loughlin, R., *J. Biol. Chem.*, 1918, xxxiii, 295.

[4] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 155; 1921, xlvii, 111, 139, 175, 235.

cereal proteins than the cereal or legume proteins have, with few exceptions, among themselves. The data in the . . . . paper illustrate the importance of animal tissues in the food supply when the diet consists mainly of such vegetable products as do not yield a mixture of proteins having a high biological value. For the special purpose of enhancing the quality of the protein in the diet they have the highest value."

TABLE I

Amino-Acid Content of Certain Proteins in Comparison with Arachin and Conarachin, the Principal Peanut Proteins.

Amino-acids.	Gliadin of wheat.	Casein of cow's milk	Beef flesh.	Arachin.*	Conarachin.*
Glycine	0.00	0.00	2.06	0.00	
Alanine	2.00	1.50	3.72	4.11	
Valine	3.34	7.20	0.81	1.13	
Leucine	6.62	9.35	11.65	3.88	
Phenylalanine	2.35	3.20	3.15	2.60	
Tyrosine	1.50	4 50	2.20	5.50	
Serine	0.13	0.50	?	?	
Cystine	0.45	0 06	?	0.85	1.07
Aspartic acid	0.58	1.39	4.51	5.25	
Glutamic acid	43.66	15.55	15.49	16.69	
Arginine	3.16	3 81	7.47	13.51	14.60
Lysine	0.92	7.61	7.59	4.98	6.04
Histidine	1.84	2.50	1.76	1.88	1.83
Proline	13.22	6.70	5.82	1.37	
Oxyproline		0.23			
Tryptophane	1.00	1.50	Present.	Present.	
Ammonia	5.22	1.61	1 07	2.03	
Totals	85.99	67.21	67.30	63.78	

* The articles quoted give no figures bearing on the relative amounts of the two peanut globulins present. Dr. D. Breese Jones has supplied me the following data from his notes, which figures lack confirmation, but probably give an approximately accurate estimate. In one experiment he obtained from 600 gm. of meal 7.5 gm. of conarachin and 27.5 gm. of arachin.

This laboratory in cooperation with the Department of Foods and Cookery has been engaged for some time past in developing a practical utilization of peanut flour in cookery. The problem of suitable recipe formulation from the view-point of palatability has been the special province of the Cookery Department and their detailed findings will appear soon. One of these that has a bearing on our work is that a blend of 75 parts of white flour to

25 parts of peanut flour is the ideal working mixture. If the peanut flour is used in excess of this amount the cooked product is tough. We had taken as our particular province the testing of recipes from the nutritive view-point and the evaluation of the meal as a white flour supplement. A short period experiment with two of the recipes demonstrated the superior growth-promoting properties of the blended flours and the results of these tests together with the recipes are given in Part I of the Experimental part of this paper. In view of the McCollum findings, however, it seemed best to conduct a long period test of the protein-supplementing value of peanut flour, controlling the experiment against muscle protein. The main purpose of the present article is to present our results in these experiments as they seem to furnish additional evidence of the high biological value of peanut proteins.

EXPERIMENTAL.

Part I.

The following recipes were worked out with peanut flour furnished us by Professor McKee of the Department of Chemical Engineering of Columbia University, and the flour was a product prepared by a commercial firm according to his specifications, but after methods capable of commercial development.

Baking Powder Biscuit.

	Ration I.	Ration II.
	gm.	*gm.*
Wheat flour (patent)	98.6	74.0
Peanut flour	0.0	24.6
Baking powder	7.0	7.0
NaCl	1.5	1.5
Butterine	17.4	17.4
Milk	60.6	60.6
Water	15.0	15.0

Yeast Rolls.

	Ration I	Ration II.
	gm	*gm.*
Wheat flour	171.2	144.0
Peanut flour	0.0	36.2
Milk	60.6	60.6
Water	53.7	53.7
NaCl	1.4	1.4
Butterine	4.9	2.4
Yeast cake	7.2	7.2

Chart 1 shows the superiority of the peanut-supplemented products as growth producers when used as the sole food of rats of the same initial age in a short test period.

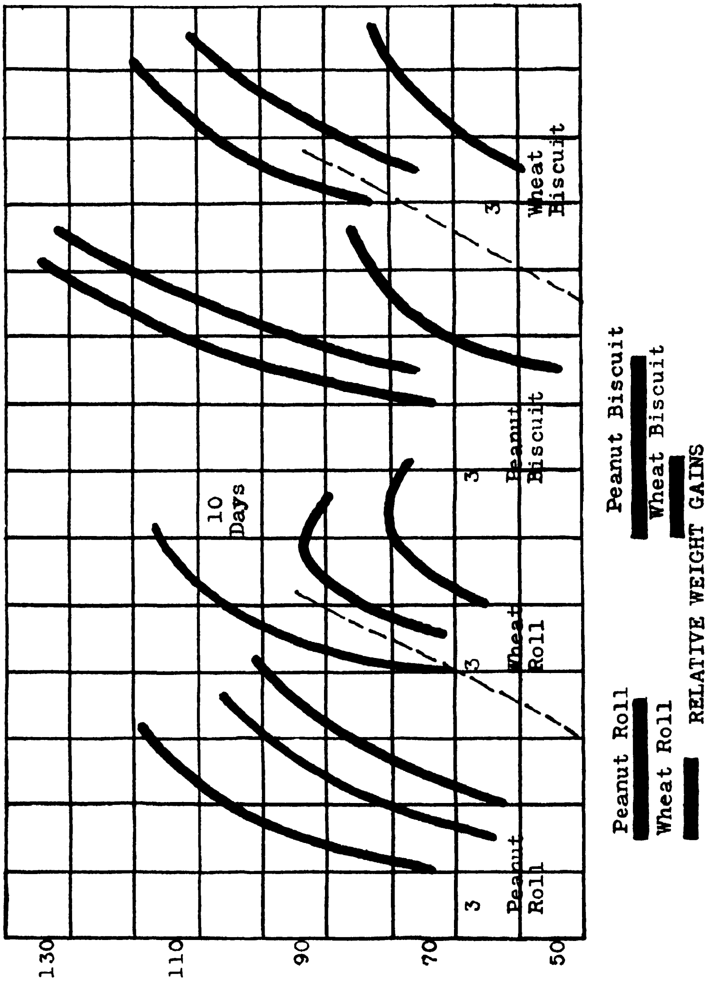

CHART 1. In a 22 day period the peanut-supplemented recipes (peanut roll and peanut biscuit) showed definite superiority as growth producers.

Part II. To Determine the Protein-Supplementing Value of Peanut Flour when Combined with White Flour as Compared with Muscle Protein.

Analyses were first made of our ration ingredients with the following results.

Ingredients.	Protein.	Fat.	Carbohydrate.	Calories.
	per cent			*per gm.*
Peanut flour	47	10.0	40	4.38
Wheat flour	11	1.1	75	3.54
Butter fat		100.0		9.00
Meat residue*	60	2.0		2.58
Starch			100	4.00
Salt Mixture 185 (McCollum)				0.00

* Our meat residue was made after the manner of Osborne and Mendel from round of beef. The first samples analyzed ran 60 per cent protein. Later preparations averaged a little higher, there being less connective tissue.

Two rations were then made up to contain approximately 9 per cent protein as follows:

Ingredients.	Ration I			Ration II.		
	Total weight	Protein.	Calories.	Total weight.	Protein	Calories.
	gm.	*gm.*		*gm.*	*gm.*	
Peanut flour	213	100	932			
Meat residue*				170	102	439
Wheat flour	1,818	200	6,436	1,818	200	6,436
Butter fat	100		900	100		900
Starch	1,079		4,316	1,122		4,488
Salt Mixture 185	123			123		
Totals	3,333	300†	12,584	3,333	302†	12,263
Calorie value per gm. of ration			3.74			3.68

* Our meat residue was made after the manner of Osborne and Mendel from round of beef. The first samples analyzed ran 60 per cent protein. Later preparations averaged a little higher, there being less connective tissue.

† The calculated values given for protein in each ration would make them approximately 9 per cent protein. Actual confirmatory analyses on the made up rations showed the value to be nearer 10 per cent protein.

It will be seen that the rations carried 3 per cent of butter fat in addition to whatever vitamine A might be in the flours, but the flours were the sole source of vitamine B. The salt mixture formed about 4 per cent of the ration. Our peanut flour averaged 47 per cent protein (N $\times$ 6.25). If Johns and Finks' factor were used (N $\times$ 5.5) the total and peanut protein of Ration I was actually lower than the amount given in the tabulation.

The experimental animals in the two series consisted of selected litters of white rats and the comparative curves are based on feeding experiments begun on the 45th day of age. Chart 2 shows the growth rates from the age of 45 days to some 390 days. On Chart 3 is given the growth curve of the best female of the peanut-wheat series together with certain representatives of each of her litters. Two of these second generation females have produced third generation offspring that are still in good condition. It will be noted, however, that while Rat A, herself, produced her first litter at the age of 85 days her offspring (Rat A_2) reached the reproductive stage considerably later. This difference may be due, we suggest, to deficiency of vitamine A. This suggestion is strengthened by results obtained following a change in certain of the rations made on the 215th day. On that day we increased the ration of butter fat from 3 to 9 per cent for certain members of the groups. The change had apparently little effect on the males, but did improve the condition of the females that received it.

When a comparison is made between the peanut-wheat rationed rats and normal rats the growth curves and reproduction records of the parent stock compare very favorably. The second and third generation rats were, however, distinctly below normal in their age-weight figures and in reproductive capacities. If, however, we compare the peanut-wheat series with the meat-wheat series the comparison is altogether in favor of the peanut as a supplement. This fact is not so apparent when we study the growth curves of the parent stocks which are nearly, if not quite, normal. But none of the second generation of meat-supplemented young lived to produce a third generation. In fact, none survived more than 31 days. Table II gives the reproduction records of the two series.

Can we conclude from the results that peanut protein is weight for weight better than meat protein in biological value? Osborne

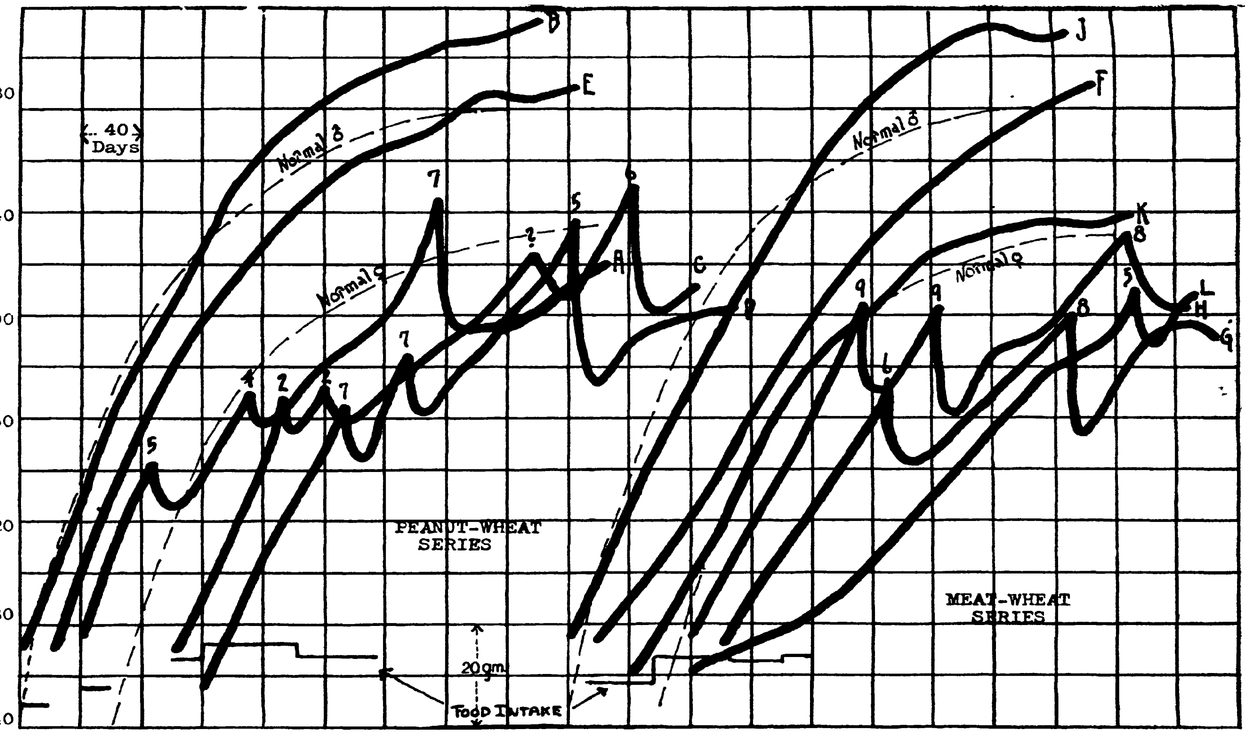

CHART 2. Growth curves of two series of rats of the same initial age fed upon a 10 per cent protein ration composed in one series of 3 per cent peanut protein and in the other of 3 per cent meat protein, the balance being provided in each case by patent wheat flour. Each curve begins with the 45th day after birth of the rat recorded. The normal curves are taken from Donaldson (Donaldson, H. H., The rat, Philadelphia, 1915).

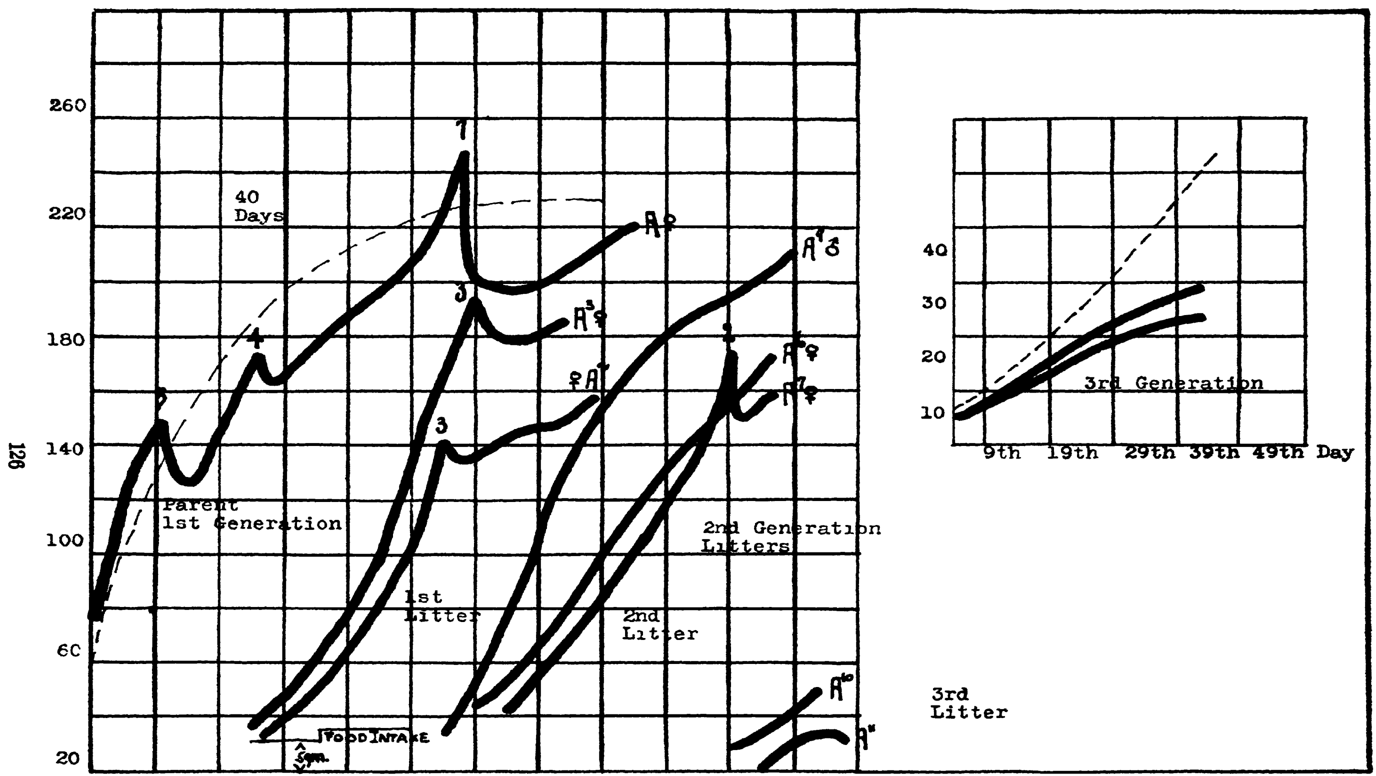

Chart 3. The parent curve is for Rat A (female) of the peanut-wheat series and a series of her offspring. With the exception of the third generation insert the curves begin with the 45th day after birth.

and Mendel have called attention to the fact that while diets may be low in protein percentage the fact that such a ration produces normal growth cannot be taken as indubitable proof of superior protein quality. They note that if the rations differ in calorie

TABLE II.

Comparative Reproduction Records.

Rat.	Series.	Litter.	Born.	Young.	Duration of life.
			day		
A	Peanut.	1	85th	5	3 died; 1 at once, 2nd at age of 84 days, and 3rd at age of 186 days. The other 2 both produced a third generation.
"	"	2	155th	4	3 lived. One female produced a third generation.
"	"	3	281st	7	3 still alive.
C	"	1	117th	2	Both died within 3 days.
"	"	2	147th	2	1 died. Other still alive.
"	"	3	279th	?	Drop in weight and floor of cage bloody in morning.
"	"	4	346th	6	All alive at date.
D	"	1	136th	7	" died within 10 days.
"	"	2	175th	7	" " oldest lived 32 days.
"	"	3	288th	5	" " " " 34 "
Total				45	15 still alive and in good health.
G	Meat.	1	159th	6	All died, oldest lived 31 days.
"	"	2	270th	8	" " " " 23 "
H	"	1	164th	9	" " " " only a few days.
"	"	2	210th	9	All died, oldest lived 30 days.
"	"	3	336th	8	" "
L	"	1	306th	2	Died at once.
"	"	2	348th	5	All died, oldest lived 14 days.
Total				47	None survived over 31 days.

value or in other appetite factors the actual daily intakes may differ widely and the actual protein consumed be different enough to account for the growth differences. The calorie values of our rations were practically the same. Records were, however, kept

of food intake over considerable part of the experiment. These figures are given in Table III.

It is difficult to draw truly quantitative comparisons from these figures. The averages are at best approximations, for in order to permit breeding several rats were together in one cage and the individual variations of intake may have been considerable. If the averages approximate the truth, however, they show that the peanut-fed series did actually consume more protein per day than the other series but that the difference is in hundredths of grams. This is not a large difference.

TABLE III.

Food Intake Records of the Two Series.

Periods.	Rats.	Series.	Food per day per rat.	Protein per day per rat.
day			*gm.*	*gm.*
45th–65th	ABCDE	Peanut.	4.60	0.46
84th–107th	"	"	7.66	0.77
139th–157th	"	"	13.23	1.32
157th–217th	"	"	15.97	1.60
217th–259th	"	"	13.60	1.36
Average			12.8	1.28
61st–101st	FGHJKL	Meat.	8.6	0.86
101st–151st	"	"	13.6	1.36
151st–201st	"	"	12.6	1.26
201st–222nd	"	"	13.7	1.37
Average			12.0	1.20
44th–94th	$A_1A_2A_3$ (A's first litter).	Peanut.	5.3	0.53
94th–145th	" " " "	"	7.5	0.75

It seems permissible, then, to grant to the peanut flour a protein quality that ranks it with meat protein for growth and it is also certain that Ration I as a whole proved markedly superior to Ration II as a provision for reproduction.

SUMMARY.

When the protein-supplementing power of peanut flour is compared with that of muscle protein by feeding rations so con-

stituted as to contain only about 10 per cent of protein, 6 to 7 per cent of this protein being contributed by wheat flour and the rest by peanut flour or meat residue, respectively, and when these rations are further supplemented with 3 per cent of butter fat, 4 per cent salts, and brought to nearly equal calorie value per gram; the peanut flour proves slightly superior to the meat as a growth producer and markedly superior for promoting reproduction.

The actual protein intakes on the two diets do not vary widely. The peanut series intake averages slightly more per day than the meat series. The calculated difference was 0.08 gm. daily. This difference does not seem sufficiently great to deny to the peanut a high biological value in its protein.

AN ACCURATE METHOD OF DETERMINING SMALL AMOUNTS OF ETHYL ETHER IN AIR, BLOOD, AND OTHER FLUIDS, TOGETHER WITH A DETERMINATION OF THE COEFFICIENT OF DISTRIBUTION OF ETHER BETWEEN AIR AND BLOOD AT VARIOUS TEMPERATURES.

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For the quantitative study of ether anesthesia there is need of a simple and accurate method for the analysis of small amounts of ethyl ether, either as the vapor in air or as the dissolved substance in water or blood. To this end such a method has been developed and is here described. Furthermore, through its utilization certain of the characteristics of ether vapor have been investigated and the relative distribution of ether between air and blood or water, in equilibrium, determined through a range of temperature sufficient for physiological purposes.

Analytical Determination of Ethyl Ether.

A number of methods have been developed for the estimation of ethyl ether vapor in air; by combustion (1), by differential absorption (2), and with the Waller balance (3). The Abbé refractometer might also be used for this purpose. All of these methods are limited in their application and for the most part are impractical under the conditions of physiological experimentation.

For the analysis of ether in blood Nicloux (4) has employed a chemical method which unfortunately requires a very generous blood specimen and involves a time-consuming distillation with chances of error.

The method here described is applicable to the determination of ether either in air or in blood. In the case of the latter a 1 ml.

sample suffices for an analysis. The principle upon which the method is based depends upon the reaction between ethyl ether and iodine pentoxide. I find that at a temperature of about 200°C. iodine pentoxide completely oxidizes ethyl ether with the liberation of 4.8 molecules of iodine for each molecule of ether. The reaction is in accordance with the equation:

$$5\ C_2H_5OC_2H_5 + 12\ I_2O_5 = 25\ H_2O + 20\ CO_2 + 24\ I$$

By collecting the liberated iodine and titrating it with thiosulfate, the amount of ether acting upon the pentoxide is determined. The general procedure does not vary materially from that employed to estimate small amounts of carbon monoxide in air. This has been used extensively in recent years, here and elsewhere (5, 6).

Apparatus.

The apparatus used for the estimation of ether is shown in Fig. 1. A suction device on the right draws a stream of air at the rate of about 200 ml. per minute through the various utensils which constitute the analyzer train. The tube on the left of the apparatus is connected to an outdoor source of air, free from contamination with carbon monoxide arising from smoke or automobile exhaust.

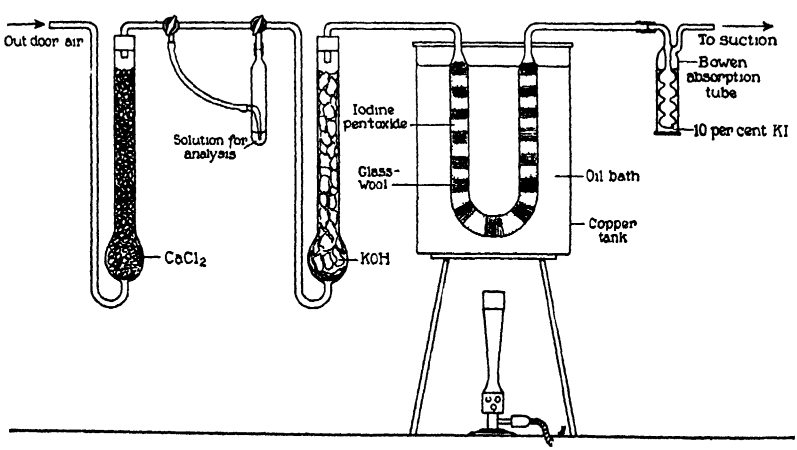

Fig. 1. Apparatus used for the determination of ethyl ether in air, blood, or other fluids.

The air is first drawn through a calcium chloride tower. Beyond this are two glass three-way cocks. This arrangement allows the shunting of the air stream through a bubbler tube holding the fluid under analysis and which is connected to the side opening of the three-way cocks. Following this is a tower filled with potassium hydroxide chips. The U-tube which holds the iodine pentoxide is immersed in a bath of oil of high boiling point (Crisco). This U-tube is of 20 mm. Pyrex glass and filled with alternate layers of glass-wool and iodine pentoxide powder to a total of about 40 gm. of the latter. The arm in the exit of this tube is tapered to make an interlocking glass joint with a Bowen absorption bottle. This joint is sealed with a short piece of rubber tubing.

Preparation of the Pentoxide.

After the apparatus has been assembled as illustrated, it is necessary to condition the pentoxide before it can be used for analytical purposes. This is accomplished by raising the temperature of the oil bath to 220 or 250°C. for several hours while a stream of air passes over the pentoxide. Considerable iodine will be driven off during this heating. Of the various preparations of iodine pentoxide available that produced by the chloric acid method is greatly to be preferred. About 15 hours of heating is sufficient to condition it thoroughly while other varieties may require an indefinitely longer time. In addition it gives lower blanks than the ordinary commercial varieties (6) which is important as even the best preparation of pentoxide decomposes to some extent spontaneously.

Method of Analysis.

During analysis the temperature of the oil bath is maintained at 200°C. While bringing it up to this temperature a stream of air is allowed to flow through the train. This materially aids in obtaining constant figures in the blank determinations which must be run with each series of analyses. When the temperature is constant the absorption bottle is charged with 10 ml. of 10 per cent potassium iodide solution and the passage of air through the train resumed for 30 minutes. At the end of this time the absorber is removed and the iodide solution carefully washed into a flask.

It is then titrated with a sodium thiosulfate solution, using starch as an indicator. The thiosulfate should be carefully standardized against a freshly prepared iodine solution. The strength of the thiosulfate is at the option of the experimenter, but I have found it expedient to prepare the solution so that 1 ml. was equivalent to 0.8233 mg. of iodine and hence to 0.1 mg. of ether. It is usually advisable to run two consecutive blank determinations. These should agree within 0.1 ml. of thiosulfate and neither should exceed 0.4 ml. of the solution suggested. Close agreement is usually found on the blank determinations. If this is not the case, however, it is necessary to repeat the procedure until constant results are obtained.

In the analysis of air containing ether vapor the receptacle holding the sample is connected between the three-way cocks of the analyzer train and the air shunted to wash out the container. If the sample for analysis is held in a mercury burette as was the case with the determinations reported in this paper, the cock of the burette is connected to the side tube of one of the three-way cocks and the sample forced into the air stream by displacement.

When the substance under examination is a liquid it is placed in a bubbler tube and the air stream shunted through it by suitable adjustment of the glass cocks. When the majority of the ether has been removed by the air stream, a beaker of water at 40°C. is placed about the tube and the air flow continues to remove the last trace of ether. When the fluid under study is blood it may be necessary to interrupt the air flow at times to allow the bubbles to settle. No volatile substance such as caprylic alcohol should be used to prevent frothing.

The total time consumed in the analytical run should be that for which the blank has been determined.

When ether has been admitted rapidly to the train the liberated iodine separates as a crystalline deposit in the arm of the U-tube connected to the absorption tube. For this reason it is advisable, near the close of the analysis, to pass the flame of a Bunsen burner along this tube to clear it of any such deposit.

On account of the very considerable amount of iodine liberated by the action of ether upon the pentoxide it is necessary to limit the amount in a sample used for analysis. The largest amount which the apparatus will handle conveniently in a single analytical run is between 5 and 6 mg. of ether.

When the run is complete the absorption bottle is removed, the solution washed into a flask and titrated as has been described for the blank determination. From the amount of thiosulfate so used the volume necessary in the blank is subtracted. The difference represents the iodine value of the ether in the sample under analysis.

Standardization of the Method.

As a means of estimating the precision of the method for the determination of ethyl ether, analyses were made on water, blood, and air containing known amounts of ether.

The ether used for the purpose of standardization was that manufactured by Squibb and Co., from which the water and alcohol were removed in the laboratory by drying over calcium oxide and redistilling to half the original volume.

A suitable amount of ether so prepared was sucked into a weighed glass ampule with long drawn capillary tips. The ends were sealed in the flame and the whole was weighed to give the amount of ether contained. The ampule was then carefully placed in a glass-stoppered bottle filled with water, the bottle closed, and the ether ampule broken by shaking it against the side of the bottle. The liberated ether quickly dissolved in the relatively large amount of water used. Samples of this solution were then analyzed for their ether content. Table I gives a summary of the data from nine determinations of this type. In no instance was the variation between the known and found amount of ether in the sample in excess of $\pm$ 0.04 mg.

The procedure as described for the analysis of known weights of ether dissolved in water was carried through with the ether dissolved in defibrinated dogs' blood. Determinations made upon the blood in which no ether had been dissolved gave readings no higher than the usual blank for the method. Appreciable amounts of acetone in the blood would interfere with the analysis of ether in the blood through the oxidation of the acetone by the pentoxide and the liberation of iodine. In fact, in the absence of ether in the blood, this method could be adapted to the very accurate estimation of acetone in this medium or in expired air.

Table II gives the comparative findings for ether dissolved in blood. The variation between the known and found amounts of

TABLE I.

Analytical Estimation of Ethyl Ether in Water Containing a Known Amount of Ether.

A solution of ether in water was made by breaking a glass ampule containing a known weight of ether in 2,680 ml. of water. 6 ml. samples were taken for analysis. Comparative findings are given.

Experiment No.	Weight of ether dissolved in water.	Weight of ether in 6 ml. sample.		Variation from known weight of ether.
		By weight.	By analysis.	
	mg.	*mg.*	*mg.*	*mg.*
1	682.4	1.528	1.560	+0.032
2	682.4	1.528	1.529	+0.001
3	682.4	1.528	1.505	−0.023
4	574.1	1.286	1.292	+0.006
5	574.1	1.286	1.275	−0.011
6	574.1	1.286	1.286	0.000
7	422.5	0.946	0.986	+0.040
8	422.5	0.946	0.977	+0.031
9	422.5	0.946	0.937	−0.009
Maximum error				±0.04
Average error				+0.009
Percentage error, maximum				±3.1 per cent

TABLE II.

Analytical Estimation of Ethyl Ether in Blood Containing a Known Amount of Ether.

A solution of ether in blood was made by breaking a glass ampule containing a known weight of ether in 252 ml. of defibrinated dogs' blood. 6 ml. samples were taken for analysis. Comparative findings are given.

Experiment No.	Weight of ether dissolved in blood.	Weight of ether in 6 ml. sample.		Variation from known weight of ether.
		By weight.	By analysis.	
	mg.	*mg.*	*mg.*	*mg.*
1	79.2	1.886	1.917	+0.031
2	79.2	1.886	1.886	0.000
3	79.2	1.886	1.915	+0.029
4	62.2	1.480	1.521	+0.041
5	62.2	1.480	1.456	−0.024
Percentage error, maximum				±2.9 per cent

ether is with a single exception, within the limits found in the analysis in water; *i.e.*, $\pm$ 0.04 mg.

In the analysis of ether dissolved in blood or water the ether is drawn from its solution into the fluid in a stream of air, and the procedure is in the ultimate the analysis of ether vapor in air. For this reason it is obvious that the method is at once available for the analysis of ether in the form of an air mixture. As a further check upon the method, however, and as a means of noting with what concordance ether vapor in air follows the laws for a true gas, mixtures of ether vapor and air were made and analyzed.

A known weight of ether was liberated in a glass receptacle by breaking a weighed ampule of ether. Diffusion was obtained by repeatedly inverting the vessel. The volume of air and ether vapor was held constant by a balanced mercury manometer and the increase in pressure due to the liberation of the ether was noted. The accuracy to which the mercury column of the manometer could be read did not exceed $\pm$ 1 mm. The temperature of the mixture was maintained uniform throughout the experiment by keeping the receptacle immersed in a large water bath. This was held at room temperature to obviate the necessity of making a temperature correction on the sample drawn for analysis. For this purpose a 10 to 20 ml. sample was removed with a mercury burette and its volume read at the prevailing barometer. This figure was then reduced to the volume occupied at the pressure within the receptacle as indicated from the manometer reading by the formula:

$$\text{Vol. at } B \times \frac{B}{B + P} = \text{Vol. occupied at } P$$

In this, B indicated the prevailing barometer in mm. and P, the pressure as read from the manometer. The analysis was carried through in the usual manner and the results, for convenience of comparison, were expressed in the weight of ether per liter of air.

As a comparison to the experimental findings the weight of ether theoretically present for the pressure observed was calculated from the gas laws by the formula:

$$\begin{array}{l}\text{Wt. in gm. per liter of} \\ \text{ether at pressure, } P, \\ \text{and temperature, } t.\end{array} = \frac{P}{760} \times \frac{\text{gm. molecular wt. of ether}}{22.4} \times \frac{273}{273+t}$$

The results of the above experiment lie within the error of the method and indicate that ether vapor in air follows the simple gas laws with sufficient conformity for physiological purposes. This was further confirmed by noting the increase in pressure in an ether vapor mixture during increase in temperature and at uniform volume. Under these conditions a true gas should behave in accord with the formula:

$$\frac{273 + t}{273 + t^1} \simeq \frac{760 + P}{760 + P^1}$$

An ether vapor-air mixture was prepared at 25°C. and gave a manometer reading of 30 mm. The temperature was then raised to 37°C. and the pressure was found to be 65 mm. The calculated increased pressure P^1 in the above formula is 66 mm.

TABLE III.

A weighed amount of ether was liberated in a glass vessel of 2,105 ml. capacity. The volume was maintained constant and the increase in pressure, P, was read by means of a balanced mercury manometer. A sample was drawn for analysis and the weight of ether found calculated on the basis of the volume occupied at the pressure within the receptacle. For convenience in reading the comparative figures are given on the basis of gm. per liter. The temperature was 25.5°C.

Experiment No.	Weight of ether liberated.	Weight of ether per liter.		Weight of ether per liter as indicated from P.	P, the pressure of the mixture as read on manometer.
		By weight.	By analysis.		
	mg.	*mg.*	*mg.*		
·1	201.8	93.21	94.00	94.92	24
2	307.2	145.94	146.10	141.60	32
3	301.1	143.52	142.09	141.60	32

Distribution of Ethyl Ether between Air and Water.

The ether equilibrium between air and blood is of extreme importance in the study of the physiology of ether anesthesia. It seemed advisable to determine first the distribution between air and water. To this end a determination has been made of the ratio of this distribution at various temperatures.

The distribution of ether between air and water was determined by equilibrating a known volume of fluid and air in the presence of a small amount of ether and determining by analysis the ether content of the two media.

For the equilibration an air sampling tube of 63.6 ml. capacity was used. This was closed at both ends by stop-cocks. The tube was calibrated with water by attaching the capillary of the lower cock by a short length of rubber tubing to a burette filled with water. The water from the burette was allowed to flow to the top of the lower stop-cock and a burette reading taken. The tube was then filled with water to the lower side of the upper cock and a second reading taken. The difference in milliliters represented the capacity of the tube between the cocks.

The tube was then carefully dried and prepared for the introduction of the water for the equilibration.

The simplest manner in which the desired amount of ether could be introduced was as a solution in the fluid used for equilibration. To this end about 0.1 ml. of ether was dissolved in 30 ml. of the fluid and this was poured into a burette with no attempt to limit the loss of ether due to diffusion during the transfer. The rubber tube from the burette was connected to the lower cock of the equilibrating tube and the fluid allowed to flow to the bottom of the lower stop-cock. A burette reading was taken and 3 ml. of fluid were run into the tube and the cock was closed. The capillary was then freed of fluid and connected to a mercury balancing bottle. Mercury was forced into the capillaries to displace the fluid held in the hole of the cock and thus bring the fluid in the tube to exactly 3 ml.

The equilibrating tube with the mercury bottle attached was then placed in a larger water bath at the desired temperature. To promote the equilibration between the fluid and the air, the tube was rotated, care being taken that the fluid was not retained at the neck of the top cock. Throughout the equilibration the pressure in the tube was maintained as atmospheric by repeatedly opening the cock which closed one end of the tube. After 20 minutes of rotation this cock was connected to the analyzer train and with the equilibration tube still in the water bath, the air displaced with mercury and analyzed for ether. When the fluid had reached the level of the upper side of the top stop-cock this was closed and the tube removed from the train. Air was then drawn into the equilibrating tube. The mercury bottle was replaced by a connection leading to fresh air and the tube shunted into the analyzer train. Both cocks were opened and the analysis of the fluid for ether was carried out in the usual manner.

In Table IV are given the results of equilibrations of air with water covering a range of temperatures from 22 to 40°C. The ratio given is expressed as relative weights of ether in fluid and air on the basis of the weight per unit of air as 1.

TABLE IV.

Distribution of Ethyl Ether between Air and Water at Various Temperatures.

3 ml. of water, 60.6 ml. of air, and approximately 5 mg. of ether were enclosed in a glass vessel and brought to equilibrium at various temperatures. The ether in the air and in the water was then determined by analysis and the ratio of distribution of the ether calculated.

Temperature.	Ether.		Ratio of ether in air to ether in water.
	Per ml. of water.	Per ml. of air.	
°C.	mg.	mg.	
21	0.650	0.021	1:30.95
26	0.777	0.031	1:25.06
32	0.795	0.042	1:18.92
37	0.703	0.045	1:15.61
38.5	0.698	0.046	1:15.15
40	0.727	0.050	1:14.54

Distribution of Ethyl Ether between Air and Blood.

A procedure similar to that used for determining the distribution of ether between air and water was employed to the end of determining the distribution between air and blood. For this purpose defibrinated dogs' blood was used. The findings are given in Table V.

Fig. 2 illustrates the curves of ether distribution for both water and blood.

The expression of ether as milligrams per unit of air is to be preferred rather than either as tension in millimeters of partial pressure, or as percentage. However, the data given are sufficient to allow a calculation to either type of expression.

For all ordinary purposes and in the range of temperature under study ether may be dealt with as a true gas. Therefore, for temperatures up to and including the boiling point of ether, 34.6°C., the formulation given on page 137 serves to convert weight per liter into mm. of pressure or *vice versa*. This can be given as

TABLE V.

Distribution of Ether between Air and Blood at Various Temperatures.

3 ml. of blood, 60.6 ml. of air, and approximately 5 mg. of ether were enclosed in a glass vessel and equilibrated at various temperatures. The ether in the air and in the blood was determined by analysis and the ratio of distribution of the ether calculated.

Temperature.	Ether.		Ratio of ether in air to ether in blood.
	Per ml. of blood	Per ml. of air.	
°*C.*	*mg.*	*mg.*	
26	0.750	0.032	1:23.40
32	0.660	0.035	1:18.85
37	0.623	0.041	1:15.20
38	0.643	0.043	1:14.95

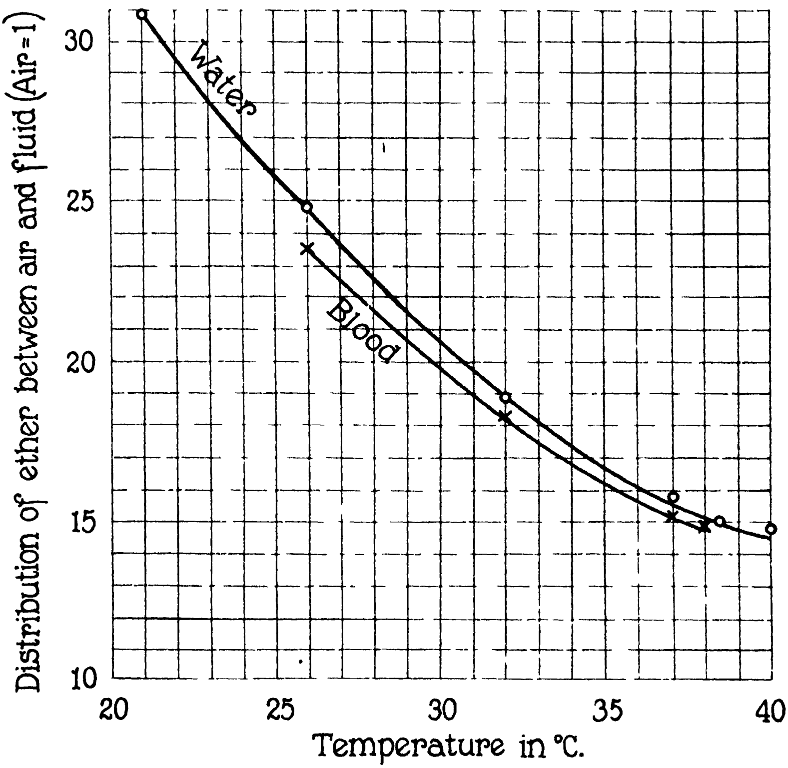

FIG. 2. Showing the relative distribution of ether between air and blood or water. The ordinates indicate the ratio, ether in fluid: ether in air, on the basis of an amount of ether in the air equal to unity.

percentage as well by multiplying the partial pressure by 100 and dividing by the prevailing barometric pressure.

When such calculations are made at a temperature over 34.6°C. and with no increase in absolute pressure, such for example, as is the case in the lungs, the weight of ether vapor per liter cannot be taken directly from the partial pressure. Under these conditions, the weight of a liter of ether vapor is that at the boiling point, times the inverse of the ratio of absolute temperature at 34.6°C. to that at higher temperature.

In calculating the effective anesthetic concentration of ether, *i.e.* the ether concentration maintained in the lungs, from mixtures of ether vapor and air made at room temperature a consideration must be given to the gas laws affecting the change in temperature and water vapor. For example, it has been estimated by Boothby (7) that the anesthetic tension of ether in man is approximately 51 mm. and for the sake of calculation we will assume the correctness of this figure. This was measured at room temperature, presumably 20°, and in air fully saturated with water vapor. Such a tension under these conditions would represent by weight 200.2 mg. of ether per liter of air. 1 liter of air at 20° would, on passing to the temperature of the body, expand by an amount indicated by the formula:

$$\text{Vol. at } t^1 = \text{Vol. at } t \times \frac{(273 + t^1)\ (760 + P^1)}{(273 + t)\ (760 + P)}$$

in which t and t^1 express the respective temperatures of the room and body and P and P^1, the partial pressure of water vapor at these two temperatures.

Thus a liter of air in passing through the change given would increase in volume to 1,045 ml. and contain 191.5 mg. of ether per liter, instead of 200.2 mg. The partial pressure exerted by the ether would become 49 mm., instead of the original 51 mm.

Blood in equilibrium with an atmosphere at 760 and containing 191.5 mg. of ether per liter would, according to the ratio given in Table V, contain 2,833 mg. of ether per liter of blood. This should express the amount in the blood during full and level anesthesia. This calculation from the work of Boothby is interesting in its comparison with the findings of Nicloux (8) from the direct determination of ether in the blood of anesthetized

dogs. According to the latter author, 1,300 to 1,400 mg. of ether per liter of blood represent deep anesthesia and 1,600 to 1,700 mg. are fatal.

It is hoped that this interesting aspect of the problem of ether anesthesia may be studied further.

SUMMARY.

1. A method for the analysis of ethyl ether in air, blood, and other fluids by means of iodine pentoxide is described. The standardization of this method is presented in detail and its accuracy indicated.

2. It is demonstrated that ethyl ether vapor follows the gas laws with sufficient conformity to allow calculation on this basis for all ordinary physiological purposes.

3. The ether distribution between air and water or blood at equilibrium has been determined through a range of temperatures varying from 24 to 40°C. A curve is presented to indicate all values between the temperatures. The curve for blood is slightly lower than that for water.

4. The necessity of applying certain fundamental corrections in calculating the anesthetic tension of ether vapor has been pointed out. On the basis of data available in the literature, an approximation has been made of the weight of ether present in the blood per liter during full anesthesia.

The author wishes to express his indebtedness to Professor Yandell Henderson for valuable suggestions and criticism.

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THE PROTEIN EFFICIENCY OF COMBINATIONS OF CORN-MEAL AND CERTAIN OTHER FEEDINGSTUFFS, NOTABLY RICE BRAN.

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There is a very great need for information which will make possible the application of the knowledge of the qualitative differences in proteins to the selection of rations for farm animals. This information must comprise such an understanding of the quality of the protein furnished by the different feedingstuffs as will show how various ones may be combined to furnish an efficient protein mixture. It is well understood that a mixture of vegetable and animal protein is of higher quality than the former alone and this knowledge is used, in feeding Omnivora; for example, the combination of corn and tankage for hogs. In fact, the value of the practise had been shown long before the reason was understood. In the case of Herbivora, however, there are no adequate guides for the selection of rations containing an efficient protein mixture. Feeding standards attempt to make the ration adequate as regards protein by specifying a much larger amount then evidently would be required were the quality high, and recommend that several sources be combined to provide this protein on the theory that a combination from several plants is likely to be of higher quality than the protein furnished by a single source. The need of more exact knowledge here is obvious.

Until such a time as adequate chemical methods are available this problem must be studied by the so called biological method, comprising growth and metabolism studies with animals. Some very definite contributions have been made to the problem from studies with farm animals, notably by Hart and coworkers. However, the use of large animals constitutes a very expensive, laborious, and time-consuming method. Although the final deci-

sion as to quality must rest on trials with the farm animal in question, and with respect to a particular function such as growth or milk production, it would seem that much preliminary study of the problem could be carried on to advantage with small animals. Such a study should be expected to furnish certain promising results on which confirmatory experiments with large animals could be based. By such a procedure some guides for selecting rations of high quality should be furnished more quickly than as though it were attempted to cover the entire range of feedingstuffs by trials with the farm animals. In accordance with this idea the studies with white rats described in this paper were undertaken.

A large number of studies have been made with white rats to ascertain the adequacy and relative quality of the proteins furnished by various sources of human food. Since many of these sources, notably the cereals, also furnish feed for animals, these studies have had a bearing on animal nutrition also, particularly, they have suggested methods for such an investigation as the present one. However, there is by no means entire agreement as to what constitutes a satisfactory method for determining the relative values of various protein sources, and the results reported by different investigators are somewhat conflicting. It appears that the selection of a method which may be relied upon to give conclusive results where the protein sources do not differ greatly as to quality, as is evidently the case with many of the feedingstuffs from vegetable sources, presents real difficulties.

Many investigators have compared protein sources on the basis of their relative abilities to cause growth or growth and reproduction without taking into consideration possible differences in food consumption. Osborne and Mendel (1) have shown that any results based on growth curves alone may be wholly misleading. On the other hand, McCollum (2) has pointed out that the fact that a rat eats according to its calorific requirements should make food intakes comparable where rations are similar as regards calorific value, under which conditions detailed records of individual intakes can add little of value. Our trials have convinced us that other factors besides calorific requirements influence food intake where rations differ markedly as regards ingredients and physical character, as frequently must be the case where different feedingstuffs are being compared. Questions of palatability and

suitability of the ration to the animal arise. Here it is necessary to know whether an individual is actually eating enough food to grow before a given ration can be listed as unsatisfactory as regards the protein factor.

Osborne and Mendel postulated that accurate comparisons could only be made where two sources were compared, with respect to the relative amounts of protein required to cause equal gains over the same period of time in rats eating substantially the same amount of food. In one study (3) the above conditions were obtained by limited feeding in accordance with increase in weight. In another study (4) data which fulfilled the conditions specified were obtained by selection from those furnished by a large number of rats fed *ad libitum* on the various sources at different planes of intake. These same investigators later described a less laborious method (5) involving *ad libitum* feeding and comparisons on the basis of gain per gram of protein eaten. A given source of protein was fed at different planes of intake in rations otherwise similar to find its maximum efficiency. The maximum figures for gain per gram so obtained for various sources and combinations were used for determining relative efficiency. It was pointed out, however, that gain per gram is an accurate measure only in rations causing fairly rapid growth, since it approaches zero as growth approaches zero.

It was planned to pattern our studies after those of Osborne and Mendel, feeding the rations *ad libitum* and then adopting either the one or the other of their recommended methods of comparison according to the data obtained. First of all, such a plan involves the feeding of rations adequate for normal growth with respect to all factors except protein. It was further recognized that all results used for comparison must come from planes of protein intake producing fairly rapid, yet not average normal growth. The necessity of fairly rapid growth has been explained. On the other hand, should growth comparable to the normal result, there would be no way of telling whether the protein intake was larger than necessary. Any protein in excess would lower the true gain per gram and cause an error thereby. It also seemed desirable that all rations be alike as regards calorific value.

Corn was the feedingstuff selected as the protein source for which supplementing sources were to be sought, because it is one

of the most largely used feeding materials, and because it is generally considered to contain proteins of poor quality. It was planned first to compare corn and various combinations with it at a single plane of protein intake and then to select the promising combinations for further study at various other planes.

EXPERIMENTAL.

Rations.

In our first series of trials a plane of protein intake of 9 per cent was used. At this plane of intake rats will grow at about two-thirds the normal rate where the protein is supplied entirely by cornmeal. On analysis of the latter it was found that 88 per cent of it was required to supply the specified protein. To this was added 5 per cent of butter to supply vitamine A and 4 per cent of the ash mixture used by Osborne and Mendel (6). The ration was then made up to 100 per cent by the addition of lard. Each ingredient was analyzed and its calorific value computed from its nutrient content. The ration was found to have 4.46 calories per gram on this basis. Various other rations were next made up in which one-third of the protein furnished by the cornmeal was supplied by other feedingstuffs to be tested as supplements. In each one of these rations the calorific value was kept identical with that of the basal ration by a proper adjustment of lard and starch content. The method of making up the rations is illustrated below:

Ration.	Ingredients.	Amount.	Protein.	Calories.
		gm.		
I	Ash mixture	4		
(Basal.)	Butter	5	0 06	45 09
	Cornmeal	88	8.96	372.65
	Lard	3		27 90
	Total	100	9.02	445 64
II	Ash mixture	4		
	Butter	5	0.06	45.09
	Cornmeal	58 64	5.97	248.32
	Linseed oil meal	7.85	2.99	33.87
	Lard	3.44		31.99
	Starch	21.07		86.39
	Total	100	9.02	445.66

Similarly, rations were made up in which one-third of the cornmeal protein was replaced by an equivalent amount from the following feedingstuffs: cottonseed oil meal, peanut oil meal, rice bran, and soy bean oil meal. It was considered that the amount of cornmeal used would furnish adequate vitamine B, and no further addendum of this vitamine was made except in a special case to be described later.

The rations were computed as shown above on a moisture-free basis. Immediately before making up a given ration moisture determinations were made on each ingredient and proper account was taken thereof in combining them.

The Rats.

The rats used were fed a composite of the experimental rations plus decreasing amounts of milk from weaning time until actually placed on experiment. This preliminary feeding period which lasted about 1 week caused a gradual accustoming of the rats to the experimental rations, and resulted in a more regular growth at the start of the experimental period than otherwise would have occurred. It also made possible the rejection of any animals not consuming the ration satisfactorily or not of normal weight for their age. The rats used were all males, weighing from 45 to 60 gm. at the start of the experimental period.

Each rat was fed as an individual, receiving its ration *ad libitum*. To avoid scattering, the feed was moistened with water and packed into the feed dish. Any food not eaten was collected, dried to a moisture-free basis weekly, and deducted from the dry matter fed. The rats were weighed weekly. As the weights for the beginning and end of the experimental period, an average of weighing on 3 successive days was taken.

It seemed desirable that the experimental period should be as long as possible without extending it into the period during which rate of growth commences to decrease markedly. The curve of average normal growth is practically a straight line up to 260 gm., indicating a regular rate of growth, but beyond this point the rate decreases increasingly rapidly, gradually approaching zero. It seemed to us that comparisons could be accurately made only during the period that a regular rate of growth would occur with

the most rapidly growing group; otherwise a rat growing as rapidly as to reach a weight at which its rate commenced to decrease would from then on be at a disadvantage as regards possible rate of growth with respect to a rat not having reached such a point because of a poorer ration. An inspection of our normal growth curve showed that at average normal growth 12 weeks was required for a rat to reach a weight of 260 gm. starting at a weight of 60 gm. Thus, 12 weeks was chosen as our experimental period, realizing that a factor of safety lay in the fact that no results were to be used for comparison where entire normal growth occurred.

Results.

The results of the first series are shown in Table I.

The vitamine B preparation added to the ration of three of the rats on cornmeal was an alcoholic extract of wheat embryo absorbed on dextrin. These three rats made up a separate trial undertaken after results had been secured, showing that rice bran protein supplemented cornmeal protein. The trial was carried out to make sure that the better results where rice bran was included were not due to a stimulation of growth caused by extra vitamine B furnished by rice bran. It is seen in the table that the rats getting the added vitamine showed no superiority.

The probable errors shown in the table were computed according to Peters' formula and are believed useful for comparing the averages in question. On the basis that a difference between two averages may be considered significant where it is at least three times its probable error, the results in Table I gave evidence that rice bran, soy bean oil meal, and peanut oil meal contain protein supplementing that of cornmeal. The evidence is not conclusive since the work of Osborne and Mendel, previously discussed, indicated that comparisons should be made after finding the plane of protein intake causing maximum gain per gram for a given source. It cannot be expected that 9 per cent is the plane producing the maximum figure for all the rations tested. For similar reasons, it must not be assumed that the combinations showing no advantage at 9 per cent are thus proved to be no more efficient than cornmeal alone.

Our next step was to study further the combinations showing promise on the basis of the results in Table I. In the first place

TABLE I.

Source of protein.	Protein in food.	Rat No.	Gain in 12 weeks.	Food intake.	Gain per gm. of protein.
	per cent		*gm.*	*gm.*	*gm.*
Cornmeal.	9.02	301	88	903	1.08
		302	100	1,052	1.05
		303	107	972	1.22
		382	66	578	1.26
		520	101	885	1.26
		504*	57	548	1.15
		518*	99	878	1.25
		519*	74	729	1.13
Average			87	818	1.18±0.023
Cornmeal and linseed oil meal.	9.02	304	81	646	1.38
		305	84	745	1.25
		306	73	612	1.32
		307	96	1,024	1.04
		308	78	802	1.08
Average			82	766	1.21±0.052
Cornmeal and cotton-seed oil meal.	9 02	309	112	1,075	1.16
		310	85	722	1.31
		311	90	835	1.19
		312	75	777	1.07
		313	95	734	1.43
Average			91	829	1.23±0.046
Cornmeal and peanut oil meal.	9 02	314	105	865	1.34
		315	147	952	1.71
		316	127	931	1.51
		317	144	1,155	1.38
		318	100	828	1.34
Average			125	946	1.46±0.052
Cornmeal and soy bean oil meal.	9.02	383	129	843	1.70
		384	138	851	1.80
		385	109	774	1.56
		386	140	888	1.75
		387	140	774	2.00
Average			131	826	1.76±0.046

* Vitamine B added.

TABLE I—*Concluded.*

Source of protein.	Protein in food.	Rat No.	Gain in 12 weeks	Food intake.	Gain per gm. of protein.
	per cent		*gm.*	*gm.*	*gm.*
Cornmeal and rice bran.	9 02	388	126	813	1.72
		389	155	995	1.73
		390	156	1,046	1.65
		391	120	881	1 51
		392	120	866	1.54
Average			135	920	1.63±0 036

it was desired to know whether these combinations were better than cornmeal alone simply because the supplements contained protein of much better quality or whether there was a mutual supplementing action. Thus, the next step was to test out rations in which soy bean oil meal, rice bran, and peanut oil meal constituted the sole source of protein. To both of the oil meals 0.1 gm. of the vitamine B preparation was added daily. This preparation contained 0.51 per cent of nitrogen. The soy bean oil meal ration did not prove suitable for some reason. At first it was eaten rather readily but after 6 or 8 weeks, consumption fell off and so little was consumed that growth practically ceased. Thus, the results were not satisfactory and are not reported. The trial with this ration furnished an example of a case where poor growth might have been erroneously attributed to poor protein, had records of food intakes not been available. In Table II are given the results with rice bran and peanut oil meal.

The results with peanut oil meal were better than those with cornmeal and equal to the combination. That the latter, containing only one-third of the protein indicated to be the better, produced as good results as where the better protein was the sole source suggests a mutual supplementing action. Such an action is more strongly indicated in the case of rice bran and cornmeal since the combination proved better than either alone. This combination was selected for further study.

It was planned to secure data on cornmeal protein, rice bran protein, and the combination of the two at such other planes of intake as the nature of the experiment would allow. The limitation here proved to be greater than was anticipated. At a 7 per

cent plane of intake a group was run satisfactorily on the combination. On the other hand, cornmeal alone at this intake produced little growth and the group on it was discontinued, since fairly rapid growth is essential if the figure for gain per gram is to be accurate, as previously discussed. However, the trial of cornmeal alone at 7 per cent showed that the figures for maximum gain per gram must lie at a plane above that percentage. At 8 per cent the individual sources and the combinations were all run satisfactorily. At 10 per cent the combination produced normal growth in the case of the majority of the rats and the results were not included, since as previously explained accurate figures

TABLE II.

Source of protein.	Protein in food.	Rat No.	Gain in 12 weeks.	Food intake.	Gain per gm. of protein.
	per cent		*gm.*	*gm.*	*gm.*
Peanut oil meal.	9.02	374	75	645	1.29
		375	93	731	1.41
		505	108	793	1.52
		506	113	802	1.56
Average.			97	743	1.45±0.046
Rice bran.	9 02	398	74	590	1.39
		399	113	731	1.71
		500	125	913	1.52
		501	79	721	1.21
		502	120	886	1.50
Average.			102	768	1.47±0.055

as to gain per gram can only result where the plane is below that required to cause normal growth. It was not possible to make a ration containing more than 9 per cent of protein from cornmeal alone and this prevented the test of this source at 10 per cent or higher. The results of these trials at various intakes, excluding those referred to as unsatisfactory are given in Table III.

It was not possible to select from the data for rice bran, cornmeal, and their combination sufficient individuals making approximately equal gains at like food intakes to make any comparison of the protein sources on such a basis. Attention was next turned to a comparison on the basis of gain per gram of protein eaten and

for this purpose the pertinent figures for the averages listed in Tables I, II, and III were assembled in Table IV.

TABLE III.

Source of protein.	Protein in food.	Rat No.	Gain in 12 weeks.	Food intake.	Gain per gm. of protein.
	per cent		*gm.*	*gm.*	*gm.*
Cornmeal and rice bran.	8.00	393	133	993	1.67
		394	150	1,063	1.76
		395	140	1,002	1.74
		396	94	707	1.66
		397	146	1,046	1.74
Average			133	962	1.71±0.017
Cornmeal and rice bran.	7.00	509	77	797	1.38
		510	71	727	1.40
		511	81	825	1.40
		529	66	874	1.08
		530	68	821	1.18
Average			73	809	1.29±0.053
Rice bran.	10.00	514	122	933	1.31
		515	96	731	1.31
		516	81	727	1.11
		517	126	954	1.32
Average			106	836	1.26±0.038
Rice bran.	8.00	521	83	803	1.29
		522	85	871	1.22
		523	59	797	0.92
		525	77	913	1.05
Average			76	846	1.12±0.066
Cornmeal.	8.00	526	62	824	0.94
		527	58	856	0.85
		528	72	930	0.97
		533	94	1,085	1.08
		534	103	1,085	1.18
Average			78	956	1.00±0.042

From a general survey of the data the superior efficiency of the combined sources seems evident. The combination produced its maximum gain per gram at 8 per cent, but the figure for 9 per cent

is not enough lower to show that the difference is a real one. The maximum for rice bran alone lay at 9 per cent. In the case of cornmeal alone the results are not as conclusive as might be desired due to the fact that a plane of intake higher than 9 per cent could not be employed. Since at all the planes tried the figure for cornmeal was always the lowest, and in view of the generally reported low value of corn protein, it seems safe to assume that at no plane of intake would cornmeal alone have produced a gain per gram equal to the maximum shown by the combination. Thus, it is concluded that the proteins of cornmeal and rice bran mutually supplement each other. The results further indicate that rice bran protein is better than that from cornmeal.

TABLE IV.

Source of protein.	Protein in food.	No. averaged.	Average gain.	Average food intake.	Average gain per gm. of protein.
	per cent		*gm.*	*gm.*	*gm.*
Cornmeal and rice bran	7.00	5	73	809	1.29±0 053
" " " "	8.00	5	135	962	1.71±0.017
"	8.00	5	78	956	1.00±0.042
Rice bran	8.00	4	76	846	1.12±0.066
Cornmeal and rice bran	9.02	5	135	920	1.63±0.036
"	9.02	8	87	818	1.18±0.023
Rice bran	9.02	5	102	768	1.47±0.055
" "	10 00	4	106	836	1.26±0.038

It may also be concluded that a combination of cornmeal and soy bean oil meal is more efficient than cornmeal alone, but decision as to whether there is a mutual supplemental action must await the result of further attempts to test soy bean oil meal alone.

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THE INORGANIC PHOSPHORUS OF THE SERUM AND PLASMA OF NINETY-ONE NORMAL ADULTS AS DETERMINED BY THE BELL AND DOISY METHOD.

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The use of the Bell and Doisy method for the determination of inorganic phosphorus in the blood has been extensive because of its comparative simplicity. Since the publication of the method, difficulties in applying the technique have been encountered, and most of the investigators who have discussed the determination have dealt primarily with these difficulties. The result has been that satisfactory series of inorganic phosphate determinations on material from normal adults have not yet been published.

The method was first described by Bell and Doisy (1) in 1920. Lehman (2) in 1921 noted that he had encountered certain difficulties in carrying out the technique on plasma. Myers and Shevky (3) in 1921 noted similar difficulties and suggested methods for overcoming them. Hess and Gutman (4) in 1922 "found it important to use a minimum quantity of oxalate." Denis and von Meysenbug (5) in 1922 stated that the oxalate used to prevent coagulation interfered with the determination, and described conditions which would give accurate values. They recommended certain changes in the strength of the solutions used in the determination, and the reduction of the anticoagulant as much as possible.

The paper of Bell and Doisy (1) contained inorganic phosphorus determinations on the blood of five normal adults. Later papers have given a number of determinations of phosphorus in the blood and plasma of infants, but have not contained information on the values for the blood of adults. Hess and Gutman (4) in 1922

published the results of thirty-two determinations on the whole blood of normal infants, and found that 4 mg. per 100 cc. were the normal value. Von Meysenbug (6) in 1922 found the value slightly higher in normal infants' sera. The average normal value of eight determinations reported by him was 5 mg. per 100 cc. of serum. These values were used as a basis for studies of the metabolism of rickets, a disease of infants and young children in which the inorganic phosphorus of the blood has been reported as abnormal by various observers.

In preparing a series of determinations upon normal adults which should serve for comparison with values found in abnormal conditions, it was believed that simultaneous determinations upon serum and oxalated plasma would be valuable. It was thought that if either series showed a markedly higher degree of constancy than the other, it would be possible to decide whether serum or plasma were the better material for study of variations in the phosphorus content due to abnormal conditions, while if the determinations agreed, a fair check upon the technique used would be furnished. The accompanying series of determinations upon the serum and plasma of ninety-one adults represents the results of the investigation.

PROCEDURE.

Blood was drawn before breakfast in a 25 cc. Luer aspirating syringe from a vein in the antecubital fossa. 15 cc. were discharged into a flask containing 30 mg. of potassium oxalate, and the remaining 10 cc. into a dry test-tube. The oxalated portion of the blood was then centrifugalized and the plasma siphoned off; in almost every specimen 5 cc. were available for the analysis. The non-oxalated portion was allowed to stand 1 hour. The clot was then broken with a fine glass rod, and the specimen was allowed to stand an additional 2 hour period. After a total of 3 hours standing, the fluid portion was decanted, centrifugalized, and the serum siphoned off. By this procedure about 4 cc. of serum were usually obtained, of which 2.5 cc. were used for the analysis. When the serum and the clot stood in contact with each other for 24 or 48 hours, high values were found for the phosphate content of the serum, but when the serum was prepared as described the results of the determinations upon different sera

were reasonably constant, and the values agreed well with those found upon the corresponding samples of plasma. Such agreement would seem to show that the values upon serum and plasma were correct, and that the concentration of potassium oxalate

TABLE I.

*Inorganic Blood Phosphorus per 100 Cc. during July and August.**

No.	Name.	Age.	Sex.	Plasma.	Serum.	Remarks.
		yrs.		*mg.*	*mg.*	
1	M.I.	21	F.	3.0	6.3	Serum 2 days old.
4	R.H.	35	M.	2.8	9.6	" 2 " "
19	H.C.	22	F.	3.2	3.2	
20	H.Y.	23	"	3.2	3.1	
21	R.W.	22	"	3.3	3.2	
22	H.M.	20	"	3.0	3.2	
23	M.T.	21	"	3.3	3.4	
46	S.W.	41	M.	2.8	3.0	
47	S.C.	44	"	2.8	3.0	
48	I.H.	61	F.	2.9	2.9	
61	E.B.	72	M.	3.0	3.0	
62	E.D.	34	F.	3.3	3.3	
63	L.B.	70	"	2.7	3.4	
64	F.S.	57	M.	2.8	3.1	
65	H.F.	80	F.	2.9	3.2	
66	K.M.	30	"	2.8	2.9	
67	S.S.	55	"	3.3	3.2	
68	I.C.	49	"	2.5	2.7	
69	T.W.	63	M.	2.8	2.9	
70	H.M.	54	"	2.5	2.8	
71	H.C.	53	"	2.7	2.9	
72	E.C.	69	"	3.0	3.0	
73	A.H.	80	F.	2.4	2.5	
74	R.B.	48	M.	3.0	3.1	
91	C.T.	67	F.	2.8	2.8	

* I wish to express my thanks to the members of the laboratory and nursing staff who acted as subjects. Lack of space does not permit insertion of the entire data. The figures given are representative results of 91 determinations.

used (2 mg. for each cc. of blood) did not interfere with the determination as carried out.

The modification of the technique recommended by Denis and von Meysenbug (5) was used with 10 cc. of the trichloroacetic

acid filtrate (plasma diluted 1: 5, serum diluted 1:10). It was found necessary to prepare the carbonate-sulfite solution every few days to prevent the formation of a brown color at the surface of the solution just before the final reading was made.

From the results presented in Table I, it appears that the range or inorganic phosphate in the plasma and serum of normal adults as determined by the Bell and Doisy method, lies between 2.5 and 3.3 mg. per 100 cc. It also appears that 2 mg. of potassium oxalate for each cubic centimeter of blood do not interfere with the determination of inorganic phosphate by this method, for the values obtained on the plasmas from such specimens are almost identical with the values found on the sera taken simultaneously if the serum and clot do not stand too long in contact with each other.

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THE EFFECT OF ECK'S FISTULA UPON PANCREATIC DIABETES IN DOGS.

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In a study of Eck's fistula, it has occurred to us that the disturbances which have been reported after this operation may be due to a derangement of the carbohydrate rather than of the protein metabolism. We have, however, never seen any very definite symptoms after Eck's fistula in dogs kept for months on a meat diet. Hawk (1) reported that the addition of extract of beef to a meat diet is more effective in producing symptoms of intoxication than is meat alone. We have tried this combination of meat and extract of beef, adding about 25 gm. of the extract to the daily ration, but this did not seem to affect our animals in any way.

Some of the more outstanding contributions to the literature of liver function deal with carbohydrate metabolism, especially in relation to the concentration of glucose in the blood. Minkowski (2) showed that glucose practically disappeared from the blood of ducks and geese after the removal of the liver. Kausch (3) found that hyperglycemia and glucosuria did not develop in pancreatectomized ducks and geese if the liver had been previously removed. Diamare (4) obtained the same results with frogs. Pavy and Siau (5) and Kaufmann (6) obtained similar results by removing the livers of dogs. These experiments were crucial, however, and obviously may not show a complete result, as the animals were probably under the influence of anesthesia throughout the entire course of the experiment. Recently, Mann (7) has been able to remove the liver from dogs in such a way that they could be observed after they recover from the influence of anesthesia. He found that the blood sugar of these dogs decreased rapidly to 0.05 per cent or less before any ill effects could be observed. If glucose were injected at this stage, the animal survived somewhat longer, even recovering for a time..

Those toxic substances which affect the liver have been found to produce hypoglycemia in so far as they have been investigated. Frank and Isaac (8) found a very definite hypoglycemia in dogs and rabbits after phosphorus poisoning. Glucose had practically disappeared from the blood of their animals within 24 hours after the phosphorus was administered. Under-

hill (9) showed that hydrazine injected subcutaneously in the form of the sulfate, causes hypoglycemia in dogs and rabbits. Underhill and Fine (10) found that hydrazine injections prevent the development of hyperglycemia and glucosuria in depancreatized dogs. Some unpublished data obtained by Bodansky show that there is a more or less marked hypoglycemia following prolonged chloroform poisoning in dogs. Underhill and Kleiner (11) showed that injections of hydrazine do not affect the nitrogen and sulfur metabolism of dogs in any important respect, indicating that protein metabolism, at least in so far as the end-products are concerned, is unchanged. The urea-forming power of the animal was found to be practically unaffected by hydrazine intoxication. The histological investigations of Wells (12) show very clearly that the changes produced by hydrazine are all to be found in the liver. He studied the tissues of both dogs and rabbits after hydrazine intoxication and found all tissues were normal except the liver, which showed a marked fatty infiltration.

Eck's fistula, in our hands, has not produced a significant decrease in the concentration of blood sugar, but then we have seen no other important changes in such animals. It is of interest to note, however, that a carbohydrate diet is said to keep Eck's fistula dogs in good health. This supports the suggestion that the function of the liver, which is diminished or destroyed by some operations of this type, is to synthesize glucose, rather than urea, as is believed by some. We are of the opinion that the livers were more extensively damaged in those animals which, in the hands of some workers, have died if kept on a meat diet. We have found no analyses of the blood sugar reported from those animals which have been unfavorably affected by this operation, therefore, this conclusion is supported only by indirect evidence.

EXPERIMENTAL.

The Eck's fistula operation on dogs was made according to the technique of Sweet (13), and in case of Dogs 7 and 30, all but a remnant of the pancreas was removed at the same time. This remnant was transplanted under the skin, then removed after a few days. In case of Dogs 8, 9, and 12, the pancreas was not removed at the time of the Eck's fistula operation but this was done some time later. The success of both operations was established by autopsy of each animal at the end of the experiment.

The dogs were kept in cages and the urine was collected in bottles containing either toluene or thymol as a preservative. The glucose in the urine was determined in the case of Dogs 7 and 8 by the Fehling titration method and in the case of Dogs 12 and 30, by the Shaffer and Hartmann (14) method. The blood sugar was determined by MacLean's (15) method. The CO_2-combining

power was determined on the whole blood by the method of Van Slyke (16). The results of urine and blood analyses are given in Tables I to V.

TABLE I.

Dog 7 (Female).

Date.	Glucose in blood.	CO_2 in blood.	Urine.	Total N in urine.	Glucose in urine.	G:N	Remarks.
1920	*per cent*	*vol. per cent*	*cc.*	*gm.*	*gm.*		
June 19							Eck's fistula, most of pancreas removed.
" 23	0.09	43.2					Remnant of pancreas removed.
" 24	0.212	43.9	750	3.77	6.00	1.59	Fasting.
" 25	0.21	47.2	420	5.06	8.4	1.66	"
" 26			365		Trace.		"
" 28	0.24	54.2	200		"		"
July 1							Fed ½ pound of lean beef.
" 3			300	3.44	1.5	0.4	Fed as above. Infected eyes.
" 4			220	2.26	1.37	0.6	Fed as above.
" 5			210	3.41	0.7	0.3	" " "
" 6							Fasting.
" 7			200		2.22		"
" 8			275		Trace.		" Eyes no longer infected.
" 15	0.31	43.4			"		Fasting.

Animal very weak and emaciated. Killed. Autopsy showed complete removal of pancreas and a perfect Eck's fistula.

DISCUSSION.

The most striking point about these animals is the marked fall of the G:N ratio from a moderately high figure to practically zero when the dogs are fasting. In the case of Dog 8 the ration was 3.5 the 2nd day after the removal of the pancreas, but gradually fell to a low figure. After 10 days, only traces of glucose appeared in the urine. Dog 7 did not have a very high G:N ratio at any time (1.66 on the 2nd day) and the glucose disappeared from the urine in a very short time when the animal was fasting. When meat was fed, there was a certain excretion of glucose, but the

G:N ratio was never higher than 0.6 at any time. Efforts were made to determine the food tolerance of these dogs. It will be seen by referring to the table that even on a meat diet, dogs excreted glucose to a greater or less extent. Dog 9 had a G:N ratio of 0.51 the 5th day after the removal of the pancreas. 250 gm. of lean beef were given on this day and a G:N ratio of 3.6 was the result. Table IV shows the result of a more extensive attempt

TABLE II.

Dog 8 (Female).

Date.	Glucose in blood.	CO_2 in blood.	Urine.	Total N in urine.	Glucose in urine.	G:N	Remarks.
1920	*per cent*	*vol. per cent*	*cc.*	*gm.*	*gm.*		
June 9							Eck's fistula.
" 23	0.08	47.9					Pancreas removed.
" 24	0.28	46.7	285	2.05	2.28	1.1	Fasting.
" 25	0.32	54.3	280	3.95	14.0	3.54	"
" 26	0.21	49.3	275		16.37		"
" 28	0.26	44.3	250	6.40	10.0	1.56	"
" 29	0.30	46.8	150	4.87	3.92	1.24	"
" 30	0.27	46.3	185	3.35	3.70	1.11	"
July 1	0.33	46.4	210	3.38	3.76	1.09	Dog ate small amount of meat.
" 2	0.31	40.3	340	4.40	6.95	1.57	Fasting.
" 3			240	2.23	1.92	0.86	"
" 4			250	3.29	1.25	0.38	"
" 5			100				"
" 6					Trace.		"
" 7			160		"		"
" 10	0.19	37.2			"		"

Animal weak and emaciated. Killed. Autopsy showed complete removal of pancreas and a perfect Eck's fistula.

to feed meat to Dog 12. The G:N ratio was fairly low when 120 gm. of meat were fed. Glucose solutions were given by injection to Dog 30 in an effort to see if the animal was really utilizing glucose. It will be seen from Table V that the glucose was excreted in the urine in quantities even greater than the amount injected. It would appear that these animals were not utilizing glucose to any great extent.

It was thought that amino-acids may have been excreted but the amount of amino nitrogen (not given in table) in the urine of Dog 30 was less than is ordinarily found in the urine of normal dogs on a usual diet. The amount of ammonia in the urine was by no means constant. At first it was relatively high, but soon fell to a normal figure, especially when the dogs were fasting. The urine was distinctly more normal in this respect, than in the case of depancreatized dogs without Eck's fistula.

TABLE III.

Dog 9 (Pregnant Female).

Date.	Glucose in blood.	CO_2 in blood.	Urine.	Total N in urine	Glucose in urine	G:N	Remarks.
1920	*per cent*	*vol. per cent*	*cc.*	*gm.*	*gm.*		
Oct. 8							Eck's fistula.
" 17	0.066						Evidence of abortion.
" 18							Pancreas removed.
" 19	0.27		520	6.4	7.45	1.16	Fasting.
" 20			830	10.3	12.57	1.22	"
" 21			870	4.72	8.7	1.87	"
" 22	0.292		490	4.61	5.34	1.15	"
" 23			275	3.19	1.65	0.51	"
" 24			280	4.06	3.68	0.9	Fed ½ pound beef.
" 25			105	1.47	5.25	3.6	Fasting.
" 26			200	3.35	5.00	1.49	"
" 28			160		1.33		Fed ½ pound beef.
" 29			200	3.08	11.1	3.6	Fed as above.
" 30			250	4.57	8.33	1.8	" " "
" 31			300	7.62	18.8	2.7	" " "
Nov. 1			350	8.89	5.39	0.6	" " "

Found dead Nov. 2. Autopsy showed abortion had occurred, also complete removal of pancreas and perfect Eck's fistula.

The CO_2-combining power of the blood was not as greatly affected as in dogs suffering from the usual type of pancreatic diabetes. This was particularly true in the case of Dogs 8 and 12. The fall in alkali reserve in the case of No. 12 was only a little more than 5 per cent in 30 days. It would seem that feeding of this animal brought about a fall in alkali reserve of the blood while fasting caused an increase. It will be noted that the CO_2-combining power of this animal on March 19 was 42.4 per cent

while on March 23 after a 3 days fast, it was 52.9 per cent, or an increase of 10.5 per cent. In none of the animals from which blood was taken, did the alkali reserve fall as rapidly as in dogs with simple pancreatectomy, as shown by Miss Crouter and one of us (17).

TABLE IV.

Dog 12 (Male).

Date.	Glucose in blood.	CO_2 in blood.	Urine.	Total N in urine.	Glucose in urine.	G:N	Remarks.
1921	*per cent*	*vol. per cent*	*cc.*	*gm.*	*gm.*		
Mar. 2							Eck's fistula.
" 8	0.106	49.97					
" 11							Pancreas removed.
" 12	0.133	47.5	1,170	6.90	6.20	0.9	Fasting.
" 13			870	5.07	11.8	2.34	"
" 14	0.24	70.4	630	4.60	9.48	2.1	"
" 15			360	3.08	5.66	1.8	"
" 16	0.24	51.6	270	3.45	4.89	1.45	"
" 17			215	4.12	4.90	1.2	Fed 120 gm. beef.
" 18			295	3.84	9.5	2.5	Fed as above.
" 19	0.28	42.4	295	5.85	7.14	1.2	" " "
" 20			520	8.65	10.15	1.2	" " "
" 22			265	3.99	3.23	0.81	
" 23	0.24	52.9	232	2.87	3.03	1.1	Fasting Mar. 21, 22, 23.
" 24			120	1.82	0.55	0.3	Fed 120 gm. beef.
" 25			185	3.62	6.15	1.7	Fed as above.
" 26			400	7.47	6.15	0.82	" " "
" 27			300	5.27	5.09	0.97	Fed 120 gm. beef.
" 28			300	7.12	9.04	1.3	Fed as above.
" 31			155	6.93	7.37	1.06	Fed 240 gm. beef.
Apr. 1	0.292	44.5	145	5.16	7.05	1.4	" 240 " "
" 2			90	4.64	4.08	0.88	Fasting.

Animal found dead Apr. 3. Autopsy showed complete removal of pancreas and perfect Eck's fistula.

That these animals were really diabetic in a certain sense, is shown by the high concentration of blood sugar. In every case examined the blood sugar increased to a definitely diabetic level. The highest amount found was in Dog 7 which had 0.31 per cent of blood sugar on one occasion. It is perhaps difficult to explain the high concentration of glucose in the blood occurring along with a

very small excretion of glucose by the kidney. It is well known that the kidneys of diabetics of long standing tend to develop a higher threshold value for glucose. It may be that this explains

TABLE V.

Dog 30 (Female).

Date.		Urine.	Total N in urine.	Glucose in urine.	G:N	Remarks.
1922		*cc.*	*gm.*	*gm.*		
May	5					Eck's fistula operation. Large portion of pancreas removed.
"	8					Remnant of pancreas removed.
"	9	1,260	7.73	28.0	3.62	Fasting.
"	10	1,330	9.37	21.5	2.3	"
"	11	920	6.48	6.12	0.94	"
"	12	815	5.45	7.44	1.36	"
"	13	1,000	4.15	6.62	1.6	"
"	14	900	3.90	7.0	1.8	"
"	15	500	2.94	4.22	1.44	"
"	16	840	2.58	3.65	1.4	"
"	17	800	3.18	4.27	1.34	"
"	18	800	3.03	4.73	1.56	"
"	19	800	2.57	1.69	0.65	"
"	20	600	2.85	3.29	1.15	"
"	21	445	1.30	0.33	0.25	"
"	22	570	2.50	0.31	0.12	"
"	23	575	2.18	0.33	0.15	Injected subcutaneously 10 gm. glucose dissolved in 50 cc. water.
"	24	880	3.11	15.99	5.14	Fasting.
"	25	1,080	4.51	9.65	2.14	"
"	26	830	2.65	3.78	1.42	"
"	27	620	3.56	1.63	0.46	"
"	28	420	3.03	0.43	0.14	"
"	29	395	2.87	1.59	0.55	Fed ½ pound beef.
"	30	560	5.30	12.52	2.36	" ½ " "
"	31	530	4.90	12.58	2.57	" ½ " "
June	1	645	6.10	21.15	3.46	Fasting.
"	2	510	3.04	3.01	0.98	"
"	3	295	1.63	0.31	0.19	"
"	4	285	1.67	0.72	0.43	"

Animal killed with chloroform June 5. Autopsy showed complete removal of pancreas. Eck's fistula had closed, but no blood was reaching the liver through the portal vein. The portal blood was apparently flowing through gastric and colonic anastomoses.

the high blood sugar accompanied by a very slight amount of glucosuria.

It was noticed that these dogs were much less subject to infection than the depancreatized dogs without Eck's fistula. The incision in the abdomen of Dog 30 became infected, and a great deal of pus was formed. Later, the wound became apparently free from infection and healed completely. The infections of the eyelids which appeared soon after the remnant of the pancreas was removed, disappeared when the G:N ratio reached a low level. These infections, both in the incision of the abdomen of Dog 30 and the eyelids of Dogs 7 and 8, reappeared more or less extensively when animals were fed as Dogs 7 and 8 or when sugar injections were made as in Dog 30. The statement is sometimes made that the increased amount of sugar in the blood is responsible for the lack of resistance to infection in diabetics. In these dogs, however, the infections cleared up even though the blood sugar remained at a definitely diabetic level. The alkali reserve did vary with the feeding, that is, it decreased in the case of Dog 12 when the animal was fed. It might be argued, therefore, that the resistance to infection is more closely associated with acidosis than with hyperglycemia. Recent work which shows the sensitiveness of tissues to very slight variations in hydrogen ion concentration may lend support to the view that acidosis is associated with a diminished resistance to infection.

We are not prepared to give any very definite conclusions from these results. But the more obvious suggestion is that considerably less than the normal amount of glucose is being formed in the body of an Eck's fistula animal than in one with normal portal circulation. Since the liver has been thrown out of the circulation to a great extent, the amino-acids from the intestine and tissues can reach the liver only in the blood which enters through the hepatic artery. They must be burned or broken down without entering the liver and it would seem from these experiments, they must be utilized to a large extent without being transformed into glucose.

As previously mentioned, several investigators have shown that the removal of the liver prevents the development of hyperglycemia and glucosuria in pancreatectomized animals. That glucose is furnished by the liver in large part at least, seems to have been

proven by Mann when he observed a marked hypoglycemia followed by death soon after the complete removal of the liver. It does not seem probable that this hypoglycemia is due only to the lack of stored carbohydrate, since it is known that the glucose in the blood does not decrease in starvation even to the point of death. Mann's observation that glucose injections restored his hepatectomized dogs even after they had become moribund, shows rather conclusively that an important function of the liver is the formation of glucose, perhaps, from amino-acids. It appears that after Eck's fistula, the amount of sugar which may be formed from protein is much less than in animals possessing a normal portal circulation.

SUMMARY.

1. It has been shown that glucose practically disappears from the urine of Eck's fistula pancreatectomized dogs after they have fasted for several days.

2. There is a definite hyperglycemia in such animals even after prolonged fasting.

3 The alkali reserve in the blood of these dogs does not decrease as rapidly as after simple pancreatectomy.

4. The ability to utilize glucose is very slight.

5. It is suggested that little glucose is formed from amino-acids in Eck's fistula dogs.

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ON THE SELECTIVE ACTION OF THE KIDNEY AS REGARDS THE EXCRETION OF INORGANIC SALTS.

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About a year ago I published (1) a series of determinations of sulfates in the blood of normal subjects and of persons suffering from a variety of pathological conditions in which it was shown that in certain cases of nephritis a considerable retention of sulfates was demonstrable by blood analysis. Since this publication, I have further investigated the retention of sulfates both in cases of clinical nephritis and in experimental nephritis in animals after the administration of salts containing this radical both by intravenous injection and by introduction into the intestine. The experimental work dealing with the latter phase of the subject is reported in this paper.

In this series of experiments solutions of various inorganic salts were injected directly into the intestine of anesthetized dogs, and analyses made on samples of serum, and in some cases on whole blood, taken at intervals over a period of several hours.

In addition I have carried out a number of experiments in which salt solutions were injected intravenously and the rapidity of excretion was followed by means of blood analyses.

The following analytical methods were employed: sodium was determined by the method of Kramer and Tisdall (2); calcium by the method by Clark (3); magnesium and sulfates by the procedures described by Denis (1, 4); chlorides by the method of Smith (5); and phosphates by that of Bell and Doisy (6).

Experiments 1 and 2, which are described below, show the results obtained by means of intestinal administration of the sulfates of magnesium and of sodium. Experiments 3 and 4, in which I have made observations on the action of sodium chloride and

sodium phosphate, respectively, were carried out as controls to the first two experiments; as in these latter cases we have one salt (sodium chloride) which is known to be rapidly absorbed from the small intestine and rapidly excreted by the kidney, whereas sodium phosphate is known to be poorly absorbed from the intestine (although according to the statements made in most text-books of pharmacology not so poorly as magnesium sulfate) and in all probability is rapidly and easily excreted by the kidney.

Experiment 1. Excretion of Magnesium Sulfate.—Male dog, weight 6.8 kilos. Nov. 3, 1921.

8.30 a.m. Morphine sulfate, 60 mg., was administered subcutaneously, ether was started at 9.40 a.m., a tracheal cannula was then inserted and a ligature tied just below the pylorus and another immediately above the ileocecal valve.

10.30 a.m. A 20 cc. sample of blood was drawn from the external jugular vein (Specimen 1) and at 10.35 a.m., 100 cc. of a warm 10 per cent solution of $MgSO_4 \cdot 7H_2O$ were injected into the tied off portion of the intestine. The intestine was then replaced within the abdominal cavity and the animal so arranged that it could be kept warm by means of an electric heater.

1.15 p.m. 20 cc. of blood were withdrawn from the right femoral vein (Specimen 2) and at 2.10 p.m. a final blood sample was taken from the external jugular vein (Specimen 3).

The three specimens of blood taken as described above were analyzed for sulfates and for magnesium; the results of these analyses are collected in Table I.

TABLE I.

Magnesium Sulfate Given by Intestine.

Specimen No.	Time after administration of $MgSO_4$.	Per 100 cc. blood.	
		SO_4 as S.	Mg
	min.	*mg.*	*mg.*
1	0	1.9	1.3
2	160	7.0	1.6
3	215	12.0	6.4

Experiment 2. Excretion of Sodium Sulfate.—Male dog, weight 9 kilos. June 4, 1922.

7.55 a.m. 60 mg. of morphine sulfate were administered subcutaneously and at 8.50 a.m. ether was started. A tracheal cannula was then inserted and the intestine was ligated as described under Experiment 1.

9.10 a.m. 20 cc. of blood were removed from the external jugular veins and at 9.30 a.m., 100 cc. of 0.5 M $Na_2SO_4 \cdot 10H_2O$ solution were injected into the intestine.

Three samples of blood were drawn during the next 3 hours as follows: 10.20 a.m., 20 cc. from the external jugular vein (Specimen 2); 11.40 a.m. from the carotid artery (Specimen 3); and 1.00 p.m. from the heart (Specimen 4).

Determinations of sodium and sulfates in these specimens of blood gave the results collected in Table II.

TABLE II.

Sodium Sulfate Given by Intestine.

Specimen No.	Time after administration of Na_2SO_4.	Per 100 cc. serum.		Per 100 cc. whole blood.	
		SO_4 as S.	Na	SO_4 as S.	Na
	min.	*mg.*	*mg.*	*mg.*	*mg.*
1	0	3.1	310	2	216
2	50	15.0	310	15	214
3	130	53.0	323	27	210
4	210	80.0	338	29	218

Experiment 3. Excretion of Sodium Chloride.—Male dog, weight 9 kilos. June 8, 1922.

Morphine and ether were administered as described under Experiments 1 and 2.

9.50 a.m. 25 cc. of blood were removed from the external jugular vein (Sample 1) and at 10.02 a.m. there were injected into the intestine, which had been previously ligated as above described, 100 cc. of a molecular solution of sodium chloride. Further, 25 cc. samples of blood were taken from the carotid artery at 10.50 a.m. (Sample 2); at 12.15 p.m. (Sample 3); and at 1.20 p.m. (Sample 4). The results of the analyses of the sera are given in Table III.

TABLE III.

Sodium Chloride Given by Intestine.

Specimen No.	Time after administration of NaCl.	Per 100 cc. serum.	
		Cl	Na
	min.	*mg.*	*mg.*
1	0	340	327
2	48	340	327
3	133	341	326
4	198	340	326

Experiment 4. Excretion of Sodium Phosphate Given by Intestine.—Male dog, weight 12 kilos. June 6, 1922.

After the administration of 60 mg. of morphine sulfate followed, after an interval of an hour, by ether, 25 cc. of blood were removed from the external jugular vein (Specimen 1). And after tying off the intestine there were introduced into this viscus at 9.20 a.m., 100 cc. of a molecular solution of disodium phosphate. Further specimens of blood were removed from the carotid artery at 10.00 a.m. (Specimen 2), at 11.00 a.m. (Specimen 3), at 12.00 m. (Specimen 4), and at 10.00 p.m. (Specimen 5).

The results obtained on analyses of the sera obtained from these specimens of blood are given in Table IV.

TABLE IV.

Sodium Phosphate Given by Intestine.

Specimen No.	Time after administration of Na_2HPO_4.	Per 100 cc. serum.	
		PO_4 as P.	Na
	min.	*mg.*	*mg.*
1	0	3.0	294
2	40	3.0	293
3	100	2.9	
4	160	2.9	294
5	760	2.9	294

Experiments 1 and 2 demonstrate the fact that a considerable increase in the sulfate content of the blood can be produced when sulfates are being absorbed from the small intestine. In Experiment 1 it would appear that a rise in the magnesium also takes place although the increase in magnesium is not sufficient in amount to balance the increase in sulfates. Experiments concerning this latter point were recorded by Hay (7) in 1882 in the course of his classical work on the saline cathartics. Hay's experimental procedure consisted in the analysis of urines collected before and after the administration of either sodium or magnesium sulfate. He believed that he was able to demonstrate the fact that more of the acid radical (SO_4) than the basic radical (Na or Mg) was excreted into the urine. This he states is due to the fact that the acid radical is absorbed more readily by the intestinal wall than is the basic part of the molecule which latter, therefore, accumulates in the lumen of the intestine. No analyses of the intestinal contents are given in proof of this hypothesis.

In Experiment 2 in which sodium sulfate was the salt under investigation results of a somewhat different nature were obtained. In the case of the sulfate radical a maximum increase of 77.9 mg. per 100 cc. of serum (an increase of about 35 per cent) was found after an absorption period of 80 minutes; and of 27 mg. per 100 cc. of whole blood (740 per cent increase) during the same time, while the sodium content of the serum increased from 310 to 338 mg. (9 per cent) and the sodium content of whole blood from 216 to 218 mg. (60.9 per cent).

I have made several other experiments of a similar nature and have invariably obtained results in agreement with the above.

In Experiments 3 and 4, in which sodium chloride and sodium phosphate, respectively, were placed in the intestine the entire absence of any accumulation of either sodium, chlorine, or phosphate in the blood would suggest two possibilities; either that the kidney is extremely permeable to chlorides and to sodium or that absorption of this salt from the intestine is very slow. In view of the well known ability of the small intestine to absorb solutions of sodium chloride the first hypothesis would appear the most tenable in the case of the salt. As a matter of fact the technique employed in these early experiments invariably leads to a consideration of the possibility of the results being more or less influenced by the relative rapidity of absorption of the various salts by the intestine, and while the general statement is made in practically all modern text-books of pharmacology that sodium chloride is absorbed from the intestine with great rapidity while sodium phosphate and sodium and magnesium sulfates are not I have felt it desirable to carry on a series of observations in which salt solutions were administered by intravenous injections.[1]

[1] It would seem that concentration of blood which has been shown by Hay (7) and by Underhill and Errico (8) to occur after the administration of saline cathartics need not be considered here in view of the magnitude of increase noted in the experiments, and as a matter of fact no attempt was made in the experiments just recorded to determine the extent of this concentration. In the case of the intravenous injections described below it was believed that the rapid introduction into the blood of a relatively large volume of fluid might result in blood dilution, but determinations of hemoglobin made on all of the experiments carried out on dogs indicated fluctuations (in blood taken 1 hour after the injection) of not more than 4 per cent, a change which may be regarded as without significance in this connection.

Experiment 5. Excretion of Magnesium Sulfate after Intravenous Injection.—Male rabbit, weight 3 kilos. Nov. 4, 1922.

The poisonous properties of magnesium sulfate, when rapidly injected, have been recognized since the pioneer experiments of Hay (7) made in 1882. As an antidote Meltzer and Auer (9) found calcium most efficient, and as in this work it was not feasible to use either subcutaneous injections of magnesium sulfate or the very slow methods of intravenous injections practised successfully by Hay and by Meltzer it was found necessary to precede the administration of magnesium sulfate (which was made as rapidly as the solution could be made to enter the veins) by an intravenous injection of calcium chloride. In all the experiments with magnesium salts in which this procedure was carried out the animals bore the rapid intravenous injection of magnesium salts without showing any of the symptoms of respiratory failure which accompany magnesium poisoning.

The details of the experiment are as follows: at 9.15 a.m. there were injected into the ear vein of the rabbit (previously anesthetized with ether) 8 cc. of a 3 per cent solution of calcium chloride, and at 9.20 a.m. 10 cc. of $\frac{1}{3}$ M solution of magnesium sulfate; 20 cc. samples of blood were removed from the carotid artery at 11.05 a.m., 12.05 p.m., and 1.00 p.m.

The results of the analyses of these samples of blood are given in Table V.

TABLE V.

Excretion of Magnesium Sulfate after Intravenous Injection.

Specimen No.	Time after administration of CaCl and $MgSO_4$.	Per 100 cc. serum.		
		SO_4 as S.	Mg	Ca
	min.	*mg.*	*mg.*	*mg.*
*		1.8	2.5	
1	105	24	3.3	
2	165	12		
3	220	12	3.0	

* The "normal" values given for SO_4 and magnesium were not obtained on this animal but are an average of analyses made on the blood of six normal rabbits.

Experiment 6. Excretion of Magnesium Sulfate after Intravenous Injection.—Male dog, weight 9.2 kilos. Nov. 16, 1922.

1 hour after the subcutaneous administration of 60 mg. of morphine sulfate the animal was anesthetized with ether and after the removal of a preliminary 25 cc. sample of blood from the carotid artery there were injected into the external jugular vein 30 cc. of 3 per cent calcium chloride solution and 15 minutes later (at 10.00 a.m.) 70 cc. of $\frac{1}{3}$ M magnesium sulfate solution were introduced into the same vein. Further samples of blood were taken at 11.00 a.m., 12.00 m., 1.00, 2.00, and 3.00 p.m. The analytical results obtained on these samples of blood are collected in Table VI.

Experiment 7. Excretion of $MgCl_2$ after Previous Intravenous Injection of Calcium Chloride.—Male dog, weight 11 kilos. Nov. 7, 1922.

The animal received 60 mg. of morphine sulfate subcutaneously, and ether 1 hour later. After removal of 30 cc. of blood from the carotid artery there were injected into the external jugular vein 30 cc. of 3 per cent calcium chloride solution followed in 15 minutes (10.05 a.m.) by 50 cc. of a $\frac{1}{3}$ M solution of magnesium chloride. Additional 25 cc. samples of blood were withdrawn at 11.00 a.m. (Sample 2), at 12.00 m. (Sample 3), at 1.00 p.m. (Sample 4), and at 2.00 p.m. (Sample 5).

TABLE VI.

Excretion of Magnesium Sulfate after Intravenous Injection.

Sample No.	Time after injecting $MgSO_4$	Per 100 cc. serum.	
		SO_4 as S.	Mg
	min.	*mg.*	*mg.*
1	0	1.6	2.0
2	60	13.0	4.8
3	120	8.1	4.8
4	180	7.6	
5	240		3.2
6	300	7.6	3.0

TABLE VII

Excretion of $MgCl_2$ after Intravenous Injection.

Sample No.	Time after administration of $CaCl_2$ and $MgCl_2$.	Per 100 cc serum.	
		Cl_2	Mg
	min.	*mg.*	*mg.*
1	0	350	2.0
2	55	350	4.0
3	115	352	4.0
4	175	352	3.0
5	235	353	2.1

The values obtained by analysis of this blood are given in Table VII.

Experiment 8. Excretion of Sodium Sulfate after Intravenous Injections.—Male dog, weight 11.5 kilos. Oct. 26, 1922.

1 hour after the subcutaneous administration of 60 mg. of morphine sulfate the animal was etherized and 25 cc. of blood were removed from the carotid artery. At 9.45 a.m. there were injected into the external jugular vein 100 cc. of $\frac{1}{3}$ M sodium sulfate solution. Further 25 cc. samples of blood were taken from the carotid artery at 10.50 a.m. (Sample 2) and at 12.55 p.m. (Sample 3).

The analytical results are given in Table VIII.

The results obtained in Experiments 5 to 8 are similar to those found in Experiments 1 and 2 and show clearly the distinct lag in the excretion of the sulfate ion. Although the fact has been repeatedly demonstrated that moderate amounts of sodium chloride can be injected intravenously without affecting in any way the sodium or chlorine content of the blood, the salt being in part rapidly excreted and its presence being in part masked by the retention of water in the body, I have carried on two experiments with sodium chloride under conditions strictly comparable to those obtaining in the experiments with sulfates.

Experiment 9. Excretion of Sodium Chloride after Intravenous Injections.—Male rabbit, 2.2 kilos. Nov. 3, 1922.

After the animal had been etherized and a preliminary sample of blood removed there were injected into the external jugular vein 30 cc. of 0.5 M sodium chloride solution. This injection was made rapidly and was complete at 9.05 a.m. At 10.05 and at 11.05 a.m. 10 cc. samples of blood were taken from the internal carotid artery. The serum obtained from the blood was removed after the latter had been allowed to stand in the refrigerator for 2 hours and was analyzed for sodium chloride. The results are given in Table IX.

Experiment 10. Excretion of Sodium Chloride after Intravenous Injections.—Male dog, weight 10.5 kilos. Nov. 9, 1922.

After the administration of 60 mg. of morphine sulfate followed 1 hour later by ether, a preliminary 25 cc. sample of blood was removed from the carotid artery at 9.10 a.m., and at 9.55 a.m. 100 cc. of 0.5 M sodium chloride were rapidly injected into the external jugular vein. Further samples of blood were taken as follows: 10.55 a.m. (Specimen 2), 11.55 a.m. (Specimen 3), and 1.10 p.m. (Specimen 4). The figures obtained on analysis of the sera obtained from these samples of blood are given in Table X.

As was to be expected my results indicate again the rapidity with which the excess of injected sodium chloride disappears from the blood, and again demonstrate the difference in behavior of the sulfate and chlorine ions.

In the following experiment I have studied the behavior of sodium phosphate on intravenous injection.

Experiment 11. Excretion of Sodium Phosphate after Intravenous Injection.—Male dog, weight 9.7 kilos. Nov. 14, 1922.

1 hour after the subcutaneous injection of 60 mg. of morphine sulfate, ether was administered and after the removal of a preliminary 30 cc. sample of blood from the carotid artery there were injected into the

TABLE VIII.

Excretion of Sodium Sulfate after Intravenous Injection.

Sample No.	Time after administration of Na_2SO_4.	Per 100 cc. serum.	
		SO_4 as S.	Na
	min.	*mg.*	*mg.*
1	0	1.2	350
2	65	20.2	348
3	190	8.9	352

TABLE IX.

Excretion of Sodium Chloride after Intravenous Injection.

Specimen No.	Time after administration of NaCl.	Per 100 cc. serum.	
		Cl	Na
	min.	*mg.*	*mg.*
1	0	354	328
2	60	352	324
3	120	356	327

TABLE X.

Excretion of Sodium Chloride after Intravenous Injection.

Specimen No.	Time after administration of NaCl.	Per 100 cc. serum.	
		Cl	Na
	min.	*mg.*	*mg.*
1	0	357	341
2	60	357	347
3	120	355	343
4	195	358	340

TABLE XI.

Excretion of Sodium Phosphate after Intravenous Injection.

Specimen No.	Time after injection of Na_2HPO_4.	Per 100 cc. serum.	
		PO_4 as P.	Na
	min.	*mg.*	*mg.*
1	0	5.0	368
2	60	4.8	366
3	120	5.0	368
4	180	4.8	360
5	240	4.9	

external jugular vein (at 11.00 a.m.) 100 cc. of a $\frac{1}{3}$ M solution of disodium monohydrogen phosphate. Further samples of blood were taken at 12.00 m. and at 1.00, 2.00, and 3.00 p.m.

The results presented in the tables demonstrate the fact that the sulfate ion is apparently excreted more slowly by the kidney than are the other ions which constitute the normal blood salts. This finding is supported by much of the earlier work on the biological properties of the sulfate ion, in which connection I may mention particularly the phenomenon which has been repeatedly demonstrated that cells in general absorb the sulfate much less readily than the chlorine ion (10) and the interesting experiments of Sollmann (11) and of Cushny (12) regarding the different behavior of the kidney in chloride and in sulfate diuresis.

A report of experiments on the intravenous injection of most of the salts used by me was published some years ago by Greenwald (13) who in his paper gives a bibliography of the earlier work in this field, and also points out the existence of a selective activity of the kidney for certain cations. This investigator made analyses of the serum of his animals before and after the injections, and in the case of some of the salts in which I obtained no accumulation, reports large increases; it must, however, be pointed out that our results are in no way contradictory as in all my experiments the dosage was small, whereas Greenwald who was engaged in a study of the toxic effects of excessive salt administration gave extremely large amounts of material and carried his experiments over a much shorter period of time than was done in this work.

SUMMARY.

Experiments made on dogs and on rabbits in which magnesium sulfate, sodium sulfate, magnesium chloride, sodium chloride, and sodium phosphate were administered by the intestine and by intravenous injection, and the subsequent excretion of these salts followed by means of blood analysis indicate a selective retention on the part of the kidney for the sulfate ion which in one case was found to accumulate in the serum to a value of 3,200 per cent of its initial concentration.

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A STUDY OF THE INORGANIC CONSTITUENTS OF THE BLOOD SERUM IN NEPHRITIS.

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Although we have for many years been in possession of data regarding the inorganic constituents of normal blood and serum chiefly through the work of Schmidt (1), Bunge (2), and Abderhalden (3), there still remain to be solved many problems regarding the concentration of these bodies in pathological conditions, both clinical and experimental. On account of the large volume of blood required, and the extremely time-consuming analytical technique used in the older work, progress in this line has been slow until the advent within recent years of the series of micro methods which have been worked out for the determination of these constituents. In this paper we wish to present the results obtained in a series of observations on the concentration of the inorganic radicals in the serum of a group of hospital patients on whom the diagnosis of nephritis or of cardiorenal disease had been made.

Many observations have, of course, been recorded of determinations of one or sometimes two or three of the inorganic constituents of nephritic sera, but so far as we have been able to learn no analyses are on record in which an attempt has been made to determine all of this class of substances in a single specimen.

While in studies of this nature it is probably desirable to obtain the blood samples in the morning when the patient is still fasting it proved impracticable in this work to adhere to this condition and our collections were therefore made in the morning from 2 to 3 hours after breakfast or in the afternoon when a similar time had elapsed since the consumption of the midday meal. In all cases the samples were allowed to stand in the refrigerator for not

more than 2 hours, the serum was then removed from the clot, and the analytical work carried out as promptly as possible. When signs of hemolysis were noted the sample was invariably rejected.

The analytical technique used was as follows: sodium and potassium by the methods of Kramer and Tisdall (4); calcium in the earlier observations was determined by the methods of Lyman (5) and of Kramer and Howland (6) and in the later samples by the technique recommended by Clark (7); magnesium by the procedure recently described by Denis (8).

For the acid radicals the following methods were used: chlorides by the procedure of Smith (9); phosphates by the Bell-Doisy method (10); and sulfates by the procedure of Denis (11).

For purposes of comparison we have also included observations on the non-protein nitrogen, creatinine, and uric acid of whole blood, all determinations of these constituents having been carried out by the procedures of Folin and Wu (12); in many cases the alkaline reserve of the plasma as determined with the Van Slyke (13) apparatus has also been included.

In considering the results presented in Table I it is first necessary to decide on what may be considered the normal concentration of the inorganic constituents in human blood.

For sodium the following figures have been published: Schmidt (1) 341 mg. for 100 cc. of serum; Wanach (14) in four subjects reported, 329, 346, 345, 352, average 339; Kramer and Tisdall (15) in seven normal adults found in all 335 mg.

For potassium the following figures are available; Schmidt in one subject reported 31 mg.; Wanach in six subjects found 21, 20, 21, 15, 31, and 33, respectively, average 23.7 mg.; Myers and Short (16) in their recent discussion of the subject of potassium retention state that the "potassium content of normal blood serum amounts to rather less than 20 mg. per 100 cc.;" Kramer and Tisdall in thirteen sera from normal adults found 19.5 mg. in every case. Four analyses made by us on normal adults gave values somewhat higher than those of Kramer and Tisdall; *viz.*, 21.0, 21.2, 22.0, and 20 mg.

The serum calcium of normal men has been found by numerous investigators (17), using the newer micro methods, to be 10 mg. per 100 cc. of blood with maximum and minimum variations of 12 and 9 mg., respectively.

For magnesium, relatively few figures are available, Schmidt in one case reported 2.8 mg. per 100 cc. of serum; Marriott and Howland (18) give their figures as varying from 2.2 to 3.5 mg.; Denis (19) has reported values varying from 1.6 to 3.5 mg.; while Kramer and Tisdall in a series of eight normal adults found from 2.3 to 4.0 mg.

Some years ago one of us (20) had occasion to carry out a considerable number of determinations of inorganic phosphate in the blood of persons who, while not strictly normal, were suffering from disorders which presumably would be without effect on phosphate metabolism. The average value obtained as a result of these observations was 2.2 mg. with maximum and minimum figures of 3.1 and 1.2 mg., respectively. Bloor's (21) results obtained by the same method on twenty-seven strictly normal persons gave slightly higher values; *i.e.*, maximum 4.3 and minimum 1.8 mg. Results obtained by us with the Bell-Doisy method, as used in the work reported in this paper, gave an average value of 2.6 mg. for nine normal subjects.

Probably the most extensive series of results on plasma chlorides has been published by McLean (22) who, in a fairly large number of normal individuals, found values varying from 570 to 620 mg. of NaCl (equivalent to 342 to 372 mg. of Cl).

The only figures as yet available for normal sulfate values are those published by Denis in 1921 (23). Additional observations have confirmed these earlier findings and make it seem probable that the normal sulfate figures for serum (or plasma) are in the neighborhood of 0.5 to 0.9 mg. of S per 100 cc. of blood.

On the whole the following figures may be taken to represent average values for the inorganic constituents of normal adult serum expressed as milligrams per 100 cc.

Na	K	Ca	Mg	Cl	PO_4 (as P)	SO_4 (as S)
335	20.5	10	2 to 4	360	3	0.5

In Table I are collected the results obtained on twenty-one sera; these cases were selected from the large amount of material available at the New Orleans Charity Hospital because in the majority of instances we were able to demonstrate marked retention of the nitrogen constituents and in some a fairly high grade

acidosis. A few cases that gave evidence of little or no retention are also included for comparison.

In several of the sera examined, Cases 52, 65, 9, and 20, it will be seen that both sodium and chlorine are notably increased above the normal values, and it should also be pointed out that in all of these four cases edema and ascites were marked. As a

TABLE I.

Inorganic Constituents of the Blood Serum in Nephritis.

Case No.	Alkaline reserve.	Per 100 cc. blood.			Per 100 cc. serum.						
		Non-protein nitrogen.	Creatinine.	Uric acid.	Na	K	Ca	Mg	Cl	Inorganic P.	SO_4 as S.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
5	40	75	3.0		354	22.0	11.0	2.2	369	5.5	
7	30	200	15.0		309	19.6	10.0	2.7	339	9.0	16.0
25	13	174	3.6		300	20.2	9.3	2.9	306	10.0	5.0
34	43	187	3.1		345	20.0	8.7	2.8	335	7.2	8.0
45		75	1.7		297	20.1	10.0	2.9	300	3.5	2.2
52	30	240	10.0	8.0	445	24.0	8.2	2.8	410	6.5	14.0
62	39	210	6.8		320	24.6	10.2	2.8	372	6.0	15.0
65		75	1.7		456	20.5	10.0		410	2.8	
72		60	1.3		361	24.0	10.9	2.5	378	3.0	
74	39	205	3.7	10	340	24.0	8.0	2.6	342	3.2	5.4
77	40	99	2.0	8	350	24.0	11.0	2.9	366	2.3	6.0
81	34	198	6.0			23.0	10.0		360	4.6	2.7
83	40	180	2.0		350	20.0	9.8	2.3	360	3.0	5.4
84	34	330	10.0		328	20.0	7.6	2.8	369	3.2	16.0
88	20	220	8.0	8.0	325	21.0	10.0	2.6	354	4.2	
90		100	1.5		354	22.0	9.0	2.9	366	2.2	
4	54	36	1.2		365	20.0	10.0	2.6	384	2.5	0.5
8	40	45	1.2		310	20.0	8.8	2.6	320	2.3	0.6
9	41	45	1.1		450	19.5	10.0	2.3	405	2.5	0.5
20		33	1.1		460	20.3	9.6	2.9	444	2.4	0.4
76		54	1.1		365	22.0	11.0	2.8	357	2.3	0.4

matter of fact the striking regularity of the sodium and chlorine concentrations found in normal sera is entirely lacking in this series, a finding which on the whole was to have been anticipated when it is considered that outside of any abnormal retention of these constituents, the fact must be taken into consideration that some of these patients had been kept for varying, and in certain

cases relatively great, lengths of time on salt-free or salt-poor diets.

In no case was there any evidence of a retention of potassium, a finding already made by Myers and Short (16).

Histories of the Patients Whose Blood Examinations Are Given in Table I.

Case 5.—Male, age 65 years, some edema of extremities, some enlargement of heart to the left. Urine shows much albumin and many fine and coarse granular casts. Diagnosis: myocarditis, chronic interstitial nephritis.

Case 7.—Male, age 27 years, marked edema and ascites. Urine very small in amount and shows many hyaline and granular casts. 6 hours before death, marked twitching of muscles over entire body. Diagnosis: acute nephritis, chronic cardiovalvular disease.

Case 25.—Male, age 25 years. Diagnosis: chronic interstitial nephritis, died 10 hours after blood was taken.

Case 34.—Male, age 36 years. Urine shows many hyaline and granular casts and much albumin. Edema practically absent. Diagnosis: acute nephritis, cardiovalvular disease, died 3 days after blood was taken.

Case 45.—Male, age 57 years. Edema of extremities, ascites, and pulmonary edema. Diagnosis: cirrhosis of liver, chronic cardiorenal disease, died a few days after blood was taken.

Case 52.—Male, age 30 years. Almost complete anuria for 3 days before death. Diagnosis: acute parenchymatous nephritis, acute splenitis (malaria).

Case 62.—Male, age 56 years. Slight edema. Urine shows much albumin and many hyaline and granular casts. Diagnosis: chronic interstitial nephritis, arteriosclerosis, died 2 days after blood was taken.

Case 65.—Male, age 54 years. Marked edema and some ascites. Diagnosis: cardiorenal disease.

Case 72.—Male, age 49 years. Marked edema. Diagnosis: cardiorenal disease.

Case 74. Male. Admitted to hospital in coma, and died a few hours afterward; no convulsions. Diagnosis: uremia.

Case 77.—Male. General edema. Diagnosis: aneurism of arch, acute and chronic nephritis.

Case 81.—Male, age 33 years. In coma after several severe convulsions. Diagnosis: uremia.

Case 83.—Male, age unknown, but probably not less than 60 years. In coma, general arteriosclerosis. Diagnosis: uremia.

Case 84.—Male, age 28 years. Edema and marked dyspnea. Diagnosis: chronic nephritis.

Case 88.—Male, age 21 years. Marked edema, in coma after several convulsions. Diagnosis: uremia.

Case 90.—Male, age 53 years. Ascites and marked edema. Diagnosis: chronic interstitial nephritis.

Case 4.—Female, age 35 years. Ascites and marked edema. Diagnosis: chronic interstitial nephritis.

Case 8.—Female, age 49 years. Some ascites and marked edema of lower extremities. Diagnosis: chronic nephritis.

Case 9.—Female, age 37 years. Marked ascites and extreme edema of the legs and feet. Diagnosis: chronic cardiorenal disease.

Case 20.—Female, age 26 years. Marked general edema. Diagnosis: chronic interstitial nephritis.

Case 76.—Male, age 35 years. Extremities very edematous. Diagnosis: mitral regurgitation, chronic interstitial nephritis.

The values for magnesium were also relatively constant, but in several cases a marked lowering of the calcium was found, and while retention of inorganic phosphate is noted in at least half the cases there appears to be no quantitative relation between the increase in the concentration of this constituent on the one hand and the decreased calcium values on the other.

From the precentage standpoint probably the most striking change in the inorganic constituents of the serum in these cases is shown in the sulfate fraction, and while it seems scarcely justifiable, in view of the small amount of experimental work which has so far been carried out, to enter into any theoretical discussion of the nature of this retention, it would seem possible that the sulfate ion occupies a position somewhat similar to that of uric acid as regards ease of excretion by the kidney, whereas the chlorine ion may be compared in this respect to the creatinine fraction; in other words the sulfate ion appears to be excreted with greater difficulty than does any other inorganic radical ordinarily present in normal blood, and, therefore, is retained more frequently and in greater amount (calculated as percentage increase over normal) than is any other inorganic constituent. The reverse may be said to be true of the chlorine ion, which on account of its apparent ease of excretion, is seldom retained except in certain (but not in all) cases where marked impairment of kidney function takes place.

Evidence has been accumulating for some years which would make the hypothesis of a selective activity on the part of the kidney for the inorganic constituents of the blood seem to rest on a relatively sure foundation. It has, of course, been long

recognized that the retention of sodium chloride is invariably accompanied by retention of water with the resultant adjustment of the osmotic pressure of the body fluids on an approximately normal level, but it is obvious that such water absorption could take care of only relatively small degrees of retention, as any large retention of sodium chloride would be incompatible with life. The relative rarity of abnormally high figures for the sodium and chlorine in the blood would therefore appear to show that these 2 ions are excreted by the kidney with great ease, whereas the retention of inorganic phosphate may amount to 400 per cent, and of sulfate to as much as 3,000 per cent of the average normal value.

SUMMARY.

A study of the inorganic constituents of the blood serum in twenty-two cases of nephritis and cardiorenal disease showed a marked increase over normal of the sodium and chlorine in only four cases (18 per cent); the inorganic phosphate fraction was increased in ten cases (45 per cent); while the inorganic sulfates were determined in only seventeen cases, eleven of which (or 64 per cent) showed increased values. Magnesium and potassium remained more or less constant, while calcium was found to be decreased in five cases (22 per cent).

If for the present we leave out of consideration calcium and magnesium, and in addition potassium, which as a constituent of the tissues may possibly be retained in some part of the body other than the serum, the contrast afforded by the behavior of the sodium and chlorine on the one hand as against phosphate and sulfate on the other affords justification for the suggestion that we have here a demonstration of the specific selective activity of the kidney towards the various normal inorganic constituents of serum, somewhat similar to the selective activity which has been shown to exist for the various non-protein constituents of blood.

Sodium and chlorine are excreted with great ease and even in the case of the badly damaged kidney, retention of these elements seldom occurs; in this respect they may be said to resemble creatinine. On the other hand, the sulfate ion is apparently excreted with difficulty so that in kidney insufficiency the concentration of this fraction may increase enormously, in some cases

to 3,000 per cent above the normal value; in this respect the sulfate ion may be likened to the uric acid fraction although the percentage increases observed are far above any concentrations of uric acid so far reported.

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THE HYDROLYSIS OF AMIDES IN THE ANIMAL BODY.

THE COMPARATIVE STABILITY OF SURFACE ACTIVE HOMOLOGS IN RELATION TO THE MECHANISM OF ENZYME ACTION.

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Steinhausen,[1] in 1914, reported a single experiment on the fate of acetamide in a phlorhizinized dog. The dog received, *per os*, a dose equivalent to 0.68 gm. of nitrogen per kilo, 72.3 per cent of which was recovered from the urine over a period of 6 days. This result, confirmed with normal cats by experiments to be reported in this paper, shows that in the animal body acetamide is hydrolyzed very slowly at the most. Schultzen and Nencki,[2] many years earlier, concluded that acetamide is not hydrolyzed at all by dogs, since it did not cause an increase in urea excretion comparable with what they found after the administration of glycine. Steinhausen's experiment shows also that this conclusion was unwarranted.

With the exception of Steinhausen, Salkowski[3] has been the only one to investigate this question by means of direct determinations of the amide excreted. Salkowski worked with rabbits, and came to a conclusion practically the opposite of that reached by Schultzen and Nencki. He decided, namely, that acetamide is mainly hydrolyzed in the rabbit, because he could recover from the urine only a small fraction of the amide given. As will be seen later, there is no doubt that amide was still being excreted when his experiments were terminated, and his estimate of the amount hydrolyzed must hence have been too low.

The present investigation was undertaken with the object of repeating Steinhausen's experiment under more nearly normal circumstances; *i.e.*, without the complication of phlorhizin. The behavior of acetamide *per se* came eventually to be only a subsidiary point, for before the work had progressed far it became evident that amide hydrolysis, being a relatively simple reaction, offered an exceptionally favorable opportunity for research on the comparative stability *in vivo* of a series of homologous compounds.

[1] Steinhausen, K., *Beitr. Physiol.*, 1914–20, i, 115.

[2] Schultzen, O., and Nencki, M., *Z. Biol.*, 1872, viii, 124.

[3] Salkowski, E., *Z. physiol. Chem.*, 1877–78, i, 1, 374.

As long as the only question involved was the behavior of acetamide given in the relatively large doses used by previous investigators, the distillation method[4] on which they all relied proved adequate for the determination of amide in urine. But a few preliminary trials with homologs of acetamide showed that sufficiently accurate comparative experiments with several members of the series could not be made without a more delicate method, for the circumstances of the case made it necessary at times to secure fairly accurate analyses when the 24 hour urine contained only a few milligrams of amide.

The accuracy needed was attained by determining the amide as ammonia instead of as fatty acid. It was shown by Fawsitt,[5] and confirmed by Werner,[6] that under certain conditions the rate of decomposition of urea by acid *falls* as the acidity is increased; the velocity of amide hydrolysis by acid, on the other hand, is roughly proportional to the acidity.[7] By properly adjusting the acidity and the time of boiling, it is consequently possible to hydrolyze all the amide in urine, leaving the urea intact (barring a small and fairly constant fraction for which a correction may be applied). The details of the method will be found in the experimental part.

Amide Hydrolysis in Vivo.

The method used here for determining the stability of amides in the living animal consisted in injecting them subcutaneously into fasting cats, the urine being analyzed in 24 hour periods as long as the excretion of amide continued.[8]

The first experiments with acetamide (Tables I and II), in which the dose was about the same as that used by Steinhausen,[1]

[4] Essentially the same as the determination of volatile fatty acid, the amide being hydrolyzed simultaneously.

[5] Fawsitt, C. E., *Z. physik. Chem.*, 1902, xli, 601.

[6] Werner, E. A., *J. Chem. Soc.*, 1918, cxiii, 84; 1920, cxvii, 1078.

[7] Kilpi, S., *Z. physik. Chem.*, 1912, lxxx, 165.

[8] In a few instances (Experiments 8, 12, and 28) complete analyses were not continued until amide entirely disappeared from the urine, since the animals were in poor condition. The excretion of amide in these experiments was, nevertheless, shown to have come to an end by analyzing urine representing a fraction of the day following the last one included in the tables.

confirm his observation that this substance remains in the body for several days, and is gradually excreted, for the most part unaltered. Propionamide likewise, given in the same amounts, is slowly excreted over a period of 4 days or more (Tables III and IV).

In Table V will be found collected the data from these four experiments, reduced to a uniform basis, together with the corresponding figures for Steinhausen's experiment and for one

TABLE I.

Acetamide (0.75 Gm. N per Kilo).

Experiment 8. Fasting cat. 2.53 kilos. 1.90 gm. amide N subcutaneously.

Day of fast.	Volume.	Total N.	Urea + NH_3-N.	Ammonia N.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total N}}$	Total S.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total S}}$	Undetermined N.	Amide N.	Remarks.
	cc.	gm.	gm.	gm.	per cent	gm.		gm.	gm.	
5	110	1.125	0.979	0.047	87.0	0.0599	16.3	0.146	0	
6	98	1.082	0.942	0.048	87.1	0.0566	16.6	0.140	0	
7	106	1.381*	0.844	0.031	61.3	0.0515	16.4	0.161	0.376	8 gm. acetamide.
8†	75	2.063	1.259	0.044	61.0	0 0524	24.0	0.176	0.628	
9	68	1.356	0.903	0 047	66.6	0.0556	16.2	0.137	0.316	
10	102	1.267	0.924	0.051	72.9	0.0585	15.8	0.127	0.216	
11	102	1.298	1.021	0.057	78.7	0.0753	13.6	0.133	0.144	
12†	30	1.012	0.838	0.056	82.8	0.0567	14 8	0.130	0.044	

* Non-protein nitrogen. The urine of this day contained 0.046 gm. of protein nitrogen, the only time that albuminuria was encountered.

† No water given.

of my own on a rabbit (given in detail in Table VI). The fate of acetamide in the rabbit is evidently much the same as in the other animals, and Salkowski's opposite conclusion must be attributed mainly to the fact that he terminated his experiments while amide was still being excreted.

A comparison of the behavior of these two amides brings out the fact that propionamide is more completely hyrolyzed in the body than acetamide. Inasmuch as an increase in molecular weight is not ordinarily accompanied by an increase in reactivity

in vitro, an examination of the comparative stability of other homologous amides in the body was undertaken, with the object of determining its relation to the molecular weight and to the rate of hydrolysis by acid and alkali in as large a series of homologs as could be used. Without varying the experimental conditions, satisfactory quantitative information could not be obtained with more than four of them (acetamide, propionamide, *n*-butyramide,

TABLE II.

Acetamide (0.6 Gm. N per Kilo).

Experiment 11. Fasting cat. 2.94 kilos. 1.76 gm. amide N subcutaneously.

Day of fast.	Volume.	Total N.	Urea + NH_3-N.	Ammonia N.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total N}}$	Total S.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total S}}$	Undetermined N.	Amide N.	Remarks.
	cc.	gm.	gm.	gm.	per cent	gm.		gm.	gm.	
4	92	1.126	0.975	0.063	86.6	0.0726	13.4	0.151	0	
5	130	1.288	1.124	0.066	87.3	0.0845	13.3	0.164	0	
6	106	1.130	0.985	0.055	87.2	0.0755	13.0	0.145	0	
7	122	1.436	0.895	0.042	62.3	0.0640	14.0	0.140	0.401	7.42 gm. acetamide.
8	90	1.446	0.978	0.044	67.7	0.0609	16.1	0.173	0.295	
9	86	1.391	1.015	0.043	73.0	0.0643	15.8	0.154	0.222	
10	110	1.297	0.965	0.048	74.4	0.0711	13.6	0.160	0.172	
11	138	1.330	1.033	0.054	77.7	0.0802	12.9	0.181	0.116	
12	102	0.994	0.824	0.050	82.9	0.0646	12.8	0.122	0.048	
13	84	0.898	0.746	0.043	83.1	0.0608	12.3	0.140	0.012	
1	84	0.898	0.746	0.043	83.1	0.0608	12.3	0.140	0.012	
15	74	0.798	0.683	0.033	85.6	0.0523	13.1	0.115	0	
16	74	0.798	0.683	0.033	85.6	0.0523	13.1	0.115	0	

and *n*-valeramide), owing to the toxicity of the higher members. The narcotic effect, as is usual in such series, increases progressively with the molecular weight; with the dose adopted (0.3 gm. of nitrogen per kilo) acetamide has no noticeable effect, while valeramide causes deep, though brief, narcosis.[9] (The larger

[9] With equivalent doses, there is in fact a progressive decrease in duration (as well as increase in intensity) of narcosis as the molecular weight rises. This suggests the interesting possibility of producing, with a single dose, any desired depth and duration of narcosis.

TABLE III.

Propionamide (0.75 Gm. N per Kilo).

Experiment 15. Fasting cat. 2.04 kilos. 1.53 gm. amide N subcutaneously.

Day of fast.	Volume.	Total N.	Urea + NH_3-N.	Ammonia N.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total N}}$	Total S.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total S}}$	Undetermined N.	Amide N.	Remarks.
	cc.	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*		*gm.*	*gm.*	
5	87	1.062	0.948	0.043	89.3	0.0717	13.2	0.114	0	
6	59	1.089	0.973	0.036	89.3	0.0698	13.9	0.116	0	
7	94	1.343	1.213	0.056	90.3	0.0876	13.8	0.130	0	
8	81	1.929	1.247	0.056	64.6	0.0866	14.4	0.189	0.493	7.98 gm. propionamide.
9	114	1.923	1.518	0.066	78.9	0.0985	15.4	0.216	0.189	
10	62	1.576	1.300	0.044	82.5	0.0912	14.2	0.195	0.081	
11	68	1.730	1.479	0.057	85.5	0.1044	14.2	0.207	0.044	
12	53	1.661	1.452	0.068	87.4	0.1064	13.6	0.209	0	

TABLE IV.

Propionamide (0.6 Gm. N per Kilo).

Experiment 13. Fasting cat. 2.56 kilos. 1.54 gm. amide N subcutaneously.

Day of fast.	Volume.	Total N.	Urea + NH_3-N.	Ammonia N.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total N}}$	Undetermined N.	Amide N.	Remarks.
	cc.	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	
4	108	0.593	0.521	0.034	87.9	0.072	0	
5}	98	0.644	0.559	0.035	86.8	0.085	0	
6}	98	0.644	0.559	0.035	86.8	0.085	0	
7	95	1.114	0.787	0.069	70.6	0.100	0.227	8.01 gm. propionamide.
8}	106	1.144	0.846	0.050	74.0	0.164	0.134	
9}	106	1.144	0.846	0.050	74.0	0.164	0.134	
10	95	1.153	0.909	0.043	78.8	0.170	0.074	
11	97	1.043	0.844	0.039	80.9	0.144	0.055	
12	101	0.790	0.652	0.034	82.5	0.104	0.034	
13	80	0.680	0.583	0.032	85.7	0.097	0	
14	100	0.655	0.569	0.029	86.9	0.086	0	

doses of the earlier experiments could not be used with the higher members, because of their greater toxicity.)

The symptoms alone give some indication of the stability of these amides in the body. The prolonged drowsiness induced by propionamide, compared with the coma of only a few hours

TABLE V.

Summary of Experiments with 0.6 to 0.75 Gm. of Amide Nitrogen per Kilo.

Animal	Rabbit.	Phlorhizinized dog.*	Cat.			
Method of administration	*Per os.*	*Per os.*	Subcutaneous.			
Diet (24 hrs.)	280 gm. carrots.	100 gm. meat.	Fasting.			
Time between beginning of period and administration of amide, *hrs*	9		1	6	5½	3
Experiment No	12		8	11	15	13
Table No	VI		I	II	III	IV
Substance	Acetamide.	Acetamide.	Acetamide.		Propionamide.	
Amide nitrogen given, *gm per kg.*	0 60	0 68	0 75	0 60	0 75	0 60
Per cent excreted:						
1st day	33.4	30.0	19.8	22.8	32.2	14.8
2nd "	23.5	19.0	33.1	16.8	12.3	8.7
3rd "	9.6	11.2	16.6	12.6	5.3	8.7
4th "	2.1	6.3	11.4	9.8	2.9	4.8
5th "	2.1	3.9	7.6	6.6		3.6
6th "		1.9	2.3	2.7		2.2
7th "				0.7		
8th "				0.7		
Total	70.7	72.3	90.8	72.7	52.7	42.8
Per cent hydrolyzed	29.3	27.7	9.2	27.3	47.3	57.2

* Data taken from Steinhausen.[1]

duration following the administration of *n*-valeramide, suggests in itself that the former remains in the tissues for a longer time. The analytical results (presented in detail in Tables VII to XI, and summarized in Table XII) bear out this interpretation, for they show an almost perfectly regular increase in rate of hydrolysis, parallel with the length of the carbon chain.

TABLE VI.

Acetamide (0.6 Gm. N per Kilo).

Experiment 12. Rabbit. 1.94 kilos. 280 gm. carrots per day. 1.16 gm. amide N *per os*.

Day.	Volume.	Total N.	Urea + NH_3-N.	Ammonia N.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total N}}$.	Undetermined N.	Amide N.	Remarks.
	cc.	*gm.*	*gm.*	*mg.*	*per cent*	*gm.*	*gm.*	
1	196	0.800	0.643	0.7	80.4	0.157	0	
2	148	0.712	0.558	0.6	78.4	0.154	0	
3	270	1.230	0.650	1.1	52.9	0.192	0.388	4.90 gm. acetamide.
4	178	1.063	0.591	0.8	55.6	0.199	0.273	
5	158	0.877	0.555	0.6	63.3	0.211	0.111	
6}	150	0.670	0.527	1.8	78.7	0.119	0.024	
7}	150	0.670	0.527	1.8	78.7	0.119	0.024	

TABLE VII.

n-Butyramide (0.3 Gm. N per Kilo).

Experiment 20. Fasting cat. 2.57 kilos. 0.771 gm. amide N subcutaneously.

Day of fast.	Volume.	Total N.	Urea + NH_3-N.	Ammonia N.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total N}}$.	Total S.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total S}}$.	Undetermined N.	Amide N.	Remarks.
	cc.	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*		*gm.*	*gm.*	
8	104	1.100	0.989	0.046	89.9	0.0655	15.1	0.111	0	
9	98	1.143	1.016	0.045	88.9	0.0653	15.6	0.127	0	
10	137	1.611	1.412	0.057	87.6	0.0797	17.7	0.141	0.058	4.79 gm. *n*-butyramide.
11	143	1.417	1.204	0.049	85.0	0.0577	20.9	0.145	0.068	
12	85	1.195	1.045	0.047	87.4	0.0574	18.2	0.135	0.015	
13	85	1.195	1.045	0.047	87.4	0.0574	18.2	0.135	0.015	
14	104	0.958	0.846	0.036	88.3	0.0543	15.6	0.112	0	
15	104	0.983	0.867	0.037	88.2	0.0546	15.9	0.116	0	
16	107	0.955	0.846	0.031	88.6	0.0541	15.6	0.109	0	

TABLE VIII.

Acetamide and n-Butyramide (0.3 Gm. N per Kilo).

Experiment 22. Fasting cat.

Day of fast.	Body weight.	Volume.	Total N.	Urea + NH_3-N.	Ammonia N.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total N}}$	Total S.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total S}}$	Undetermined N.	Amide N.	Remarks.
	kg.	*cc.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*		*gm.*	*gm*	
8		100	0.918	0.788	0.038	85.8	0.0581	13.6	0.130	0	
9		109	0.964	0.818	0.039	84.8	0.0607	13.5	0.146	0	
10		115	0.884	0.756	0.036	85.5	0.0567	13.3	0.128	0	
11}		105	0.838	0.716	0.038	85.4	0.0539	13.3	0.122	0	
12}		105	0.838	0.716	0.038	85.4	0.0539	13.3	0.122	0	
13	1.82	155	1.066	0.748	0.038	70.2	0.0548	13.7	0.126	0.192	2.30 gm. acetamide subcutaneously (0.546 gm. N).
14		100	0.853	0.662	0.039	77.6	0.0461	14.4	0.101	0.090	
15		94	0.719	0.594	0.036	82.6	0.0434	13.7	0.089	0.036	
16		106	0.709	0.600	0.037	84.6	0.0428	14.0	0.093	0.016	
17		104	0.713	0.619	0.037	86.8	0.0450	13.8	0.085	0.009	
18		108	0.702	0.612	0.035	87.2	0.0447	13.7	0.090	0	
19}		93	0.712	0.627	0.040	88.1	0.0466	13.5	0.085	0	
20}		93	0.712	0.627	0.040	88.1	0.0466	13.5	0.085	0	
21}		90	0.683	0.600	0.034	87.8	0.0432	13.9	0.083	0	
22}		90	0.683	0.600	0.034	87.8	0.0432	13.9	0.083	0	
23	1.57	122	1.179	0.973	0.082	82.5	0.0621	15.7	0.121	0.085	2.93 gm. n-butyramide subcutaneously (0.471 gm. N).
24		112	0.796	0.659	0.026	82.8	0.0378	17.4	0.112	0.025	
25		87	0.638	0.533	0.021	83.5	0.0342	15.6	0.091	0.014	
26		90	0.659	0.567	0.028	86.0	0.0364	15.6	0.092	0	
27		92	0.600	0.521	0.025	86.8	0.0390	13.4	0.079	0	
28		92	0.600	0.521	0.025	86.8	0.0390	13.4	0.079	0	

TABLE IX

n-Valeramide and Acetamide (0.3 Gm. N per Kilo).

Experiment 28. Fasting cat.

Day of fast.	Body weight.	Volume	Total N.	Urea + NH_3-N.	Ammonia N.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total N}}$	Total sulfate S	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total sulfate S}}$	Undetermined N	Amide N.	Remarks.
	kg.	cc	gm	gm	gm	per cent	gm		gm.	gm.	
4		86	1.132	1.027	0.045	90.7	0.0581	17.7	0.105	0	
5}		96	1.014	0.925	0.044	91.2	0.0533	17.4	0.089	0	
6}		96	1.014	0.925	0.044	91.2	0.0533	17.4	0.089	0	
7	1.55	91	1.194	1.051	0.061	88.0	0.0493	21.3	0.122	0.021	3.35 gm. *n*-valeramide subcutaneously (0.465 gm. N).
8		142	1.497	1.283	0.0t2	85.7	0.0512	25.1	0.214	0	
9}		101	0.905	0.789	0.036	87.2	0.0454	17.4	0.116	0	
10}		101	0.905	0.789	0.036	87.2	0.0454	17.4	0.116	0	
11}		91	0.830	0.745	0.031	89.8	0.0459	16.2	0.085	0	
12}		91	0.830	0.745	0.031	89.8	0.0459	16.2	0.085	0	
13	1.31	118	1.107	0.861	0.030	77.8	0.0520	16.6	0.112	0.134	1.66 gm. acetamide subcutaneously (0.394 gm. N).
14		83	0.901	0.752	0.027	83.5	0.0433	17.4	0.088	0.061	
15}		80	0.909	0.787	0.030	86.6	0.0498	15.8	0.093	0.029	
16}		80	0.909	0.787	0.030	86.6	0.0498	15.8	0.093	0.029	
17}		82	1.474	1.305	0.045	88.5	0.0817	16.0	0.160	0.009	
18}		82	1.474	1.305	0.045	88.5	0.0817	16.0	0.160	0.009	

TABLE X.

Propionamide and n-Valeramide (0.3 Gm. N per Kilo).

Experiment 34. Fasting cat.

Day of fast.	Body weight.	Volume.	Total N.	Urea + NH_3-N.	Ammonia N.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total N}}$	Total sulfate S.	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total sulfate S.}}$	Undetermined N.	Amide N.	Remarks.
	kg.	*cc.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*		*gm.*	*gm.*	
6		90	1.225	1.059	0.052	86.5	0.0504	21.0	0.166	0	
7		86	1.186	1.040	0.050	87.7	0.0524	19.8	0.146	0	
8		84	1.144	1.006	0.046	87.9	0.0520	19.3	0.138	0	
9	2.48	120	1.534	1.253	0.043	81.7	0.0593	21.1	0.165	0.116	3.88 gm. propionamide subcutaneously (0.744 gm. N).
10		101	1.351	1.085	0.045	80.3	0.0371	29.2	0.126	0.140	
11		75	1.009	0.849	0.038	84.1	0.0391	21.7	0.102	0.058	
12		65	0.981	0.846	0.041	86.2	0.0452	18.7	0.117	0.018	
13		70	1.020	0.894	0.038	87.6	0.0516	17.3	0.126	0	
14		69	1.085	0.964	0.043	88.9	0.0564	17.1	0.121	0	
15		80	1.101	0.972	0.040	88.3	0.0540	18.0	0.129	0	
16	2.25	78	1.534	1.342	0.065	87.5	0.0534	25.1	0.165	0.027	4.87 gm. *n*-valeramide subcutaneously (0.675 gm. N).
17		96	1.391	1.166	0.037	83.8	0.0367	31.8	0.225	0	

TABLE XI.

Propionamide (0.3 Gm. N per Kilo).

Experiment 29. Fasting cat. 1.39 kilos. 0.416 gm. amide N subcutaneously.

Day of fast.	Volume.	Total N.	Urea + NH_3-N.	Ammonia N.	$\frac{\text{Urea + NH}_3\text{-N}}{\text{Total N}}$	Total sulfate S.	$\frac{\text{Urea + NH}_3\text{-N}}{\text{Total sulfate S}}$	Undetermined N.	Amide N.	Remarks.
	cc.	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*		*gm.*	*gm.*	
5	106	1.205	1.073	0.061	89.0	0.0635	16.9	0.132	0	
6	110	1.232	1.088	0.058	88.3	0.0685	15.9	0.144	0	
7	125	1.488	1.189	0.068	79.9	0.0605	19.7	0.197	0.102	2.17 gm. propion-
8	122	1.362	1.125	0.060	82.6	0.0645	17.4	0.167	0.070	amide.
9 }	100	1.286	1.118	0.060	86.9	0.0744	15.0	0.164	0.004	
10 }	100	1.286	1.118	0.060	86.9	0.0744	15.0	0.164	0.004	
11 }	109	1.385	1.219	0.060	88.0	0.0836	14.6	0.166	0	
12 }	109	1.385	1.219	0.060	88.0	0.0836	14.6	0.166	0	

TABLE XII.

Summary of Experiments with 0.3 Gm. of Amide Nitrogen per Kilo Given Subcutaneously to Fasting Cats.

Time between beginning of period and injection of amide, *hrs* ...	3½	0	1½	5	½	½	½	4
Experiment No	22	28	29	34	20	22	28	34
Table No	VIII	IX	XI	X	VII	VIII	IX	X
Substance	Acetamide		Propion-amide.		*n*-Butyr-amide		*n*-Valer-amide.	
Per cent excreted:								
1st day	35.2	34.0	24.5	15.6	7.5	18.0	4.5	4.0
2nd "	16.5	15.5	16.8	18.8	8.8	5.3		
3rd "	6.6	7.4 }	1.0 }	7.8	1.9 }	3.0		
4th "	2.9	7.4 }	1.0 }	2.4	1.9 }			
5th "	1.6	2.3 }						
6th "		2.3 }						
Total	62.8	68.9	43.3	44.6	20.1	26.3	4.5	4.0
Per cent hydrolyzed	37.2	31.1	56.7	55.4	79.9	73.7	95.5	96.0
Average	34.2		56.1		76.8		95.8	

The mere fact that the total amount of amide excreted diminishes as the molecular weight rises does not necessarily show that the rate of hydrolysis follows the same order. The residual amide, not recovered from the urine as such, must be shown to have been hydrolyzed, and the usual procedure in such cases is to determine the amount of urea (plus ammonia) excreted, using the sulfur output as a control. The ratio of urea + ammonia nitrogen to total sulfur or to total sulfate[10] in the urine of cats usually becomes fairly constant (within about 5 per cent) after the first few days of fasting (generally at a level of about 13 in the one case, or 15 in the other). Deviations, however, occur, and the method is not especially accurate under the conditions of these experiments, where the amount of amide administered is necessarily rather small, and the rate of extra urea production often slow. The method, nevertheless, suffices to show decided differences in urea production among the four amides used. The extra urea + ammonia nitrogen produced in each of the experiments included in Table XII, calculated on as uniform a basis as possible, is given below in grams per kilo of body weight:

Acetamide	0.05, 0.04
Propionamide	0.22, 0.17
n-Butyramide	0.30, 0.26
n-Valeramide	0.38, 0.38

While the absolute figures are in most instances too high (except in the case of acetamide), they are parallel with the molecular weight, and within the expected limit of error they show that the amide not excreted as such has been hydrolyzed and excreted as urea (plus ammonia).

This conclusion is confirmed also by the course of the amide excretion from day to day. If the higher amides were less readily excreted, and not more readily hydrolyzed, it is not likely that their excretion would fall off, if anything, *more* abruptly than in the case of the lower members, or that it would be finished in a shorter time.

It appears, then, to be reasonably certain that the velocity of hydrolysis of these four homologs increases with the molecular

[10] Total sulfur was used in the earlier experiments, and total sulfate later. The advantage of the latter is that it corresponds more nearly with urea from the standpoint of metabolic origin.

weight. This would be of no particular consequence if the order of stability were the same *in vitro* in the presence of ordinary hydrolyzing agents, but that is not the case. The most reliable comparative measurements of the rates of hydrolysis of the amides of the lower fatty acids (by acid and alkali) are those of Crocker.[11] Since he did not examine *n*-valeramide, its stability in solutions of hydrochloric acid and of sodium hydroxide has now been determined in relation to that of *n*-butyramide (see Experimental part for details). The velocity constants for these two amides, obtained under uniform conditions, are recorded in Table XIII,

TABLE XIII.

Velocity Constants of Amide Hydrolysis by Acid and Alkali.

Amide.	Hydrochloric acid.				Sodium hydroxide.			
	63 2°C.*		100°C.		63 2°C.†		100°C.	
	Concentration.	k‡	Concentration (approximate).	k	Concentration.	k‡	Concentration (approximate).	k
Acetamide.	M	0.0163			0.25M	0.0401		
Propionamide.	M	0.0199			0.25M	0.0351		
n-Butyramide.	0.75M	0.0096	0.1M	0.231	0.25M	0.0143	0.1M	0.129
n-Valeramide.			0.1M	0.239			0.1M	0.105

* Crocker.[11]

† Crocker and Lowe.[11]

‡ In Crocker's experiments, the amide and HCl (or NaOH) were present in equivalent concentrations, hence $k = \frac{1}{t} \cdot \frac{x}{a(a-x)}$.

into which representative data from Crocker's experiments with the three lower members have been incorporated.

When hydrolyzed by acid, propionamide is somewhat less stable than acetamide, while butyramide and valeramide react at practically equal rates, but considerably more slowly than the lower members. This order of stability is quite different from that found *in vivo*. The mechanism of amide hydrolysis in the body, aside from the steps in which enzymes are involved, is probably more nearly analogous to hydrolysis by alkali (rather

[11] Crocker, J. C., *J. Chem. Soc.*, 1907, xci, 593. Crocker, J. C., and Lowe, F. H., *J. Chem. Soc.*, 1907, xci, 952.

than acid) in the test-tube, since the body fluids are alkaline, and especially in view of the similarities between the biochemical cleavage of glucose and its behavior in alkaline solution outside the body.[12] If that is so, the order of stability for these four amides in the living animal is precisely the reverse of the order *in vitro*, where the rate of hydrolysis by alkali falls progressively as the molecular weight rises.

The existence of a similar relationship between molecular weight and stability in enzyme reactions with surface active substrates is more or less evident in a number of other cases. It is well defined over a limited range in the case of ester hydrolysis by lipase in the test-tube, while in the few cases in which the stability of homologs in the living animal has previously been investigated the available facts at least suggest a similar interpretation. These *in vivo* reactions, however, are all complex oxidations which cannot at present be imitated in the absence of enzymes, and until that is possible they cannot have the same significance in this connection as amide hydrolysis.

Ester Hydrolysis in Vitro.

Kastle and Loevenhart[13] observed that the rate of hydrolysis of ethyl acetate, ethyl propionate, and ethyl butyrate by pancreatic and hepatic lipase increases with the molecular weight. They were particularly impressed by this finding (which has since been confirmed[14,15] under more adequately controlled conditions, *i.e.*, with all three esters completely dissolved) because ethyl butyrate is much less readily hydrolyzed by acid than either of the other esters.[16,17,18] In Table XIV have been gathered a number of similar cases in which lipase modifies the order of stability found by using acid as the catalyst. In some series, the

[12] Woodyatt, R. T., *J. Biol. Chem.*, 1915, xx, 129.

[13] Kastle, J. H., and Loevenhart, A. S., *Am. Chem. J.*, 1900, xxiv, 491.

[14] Kastle, J. H., *Bull. Hyg. Lab., U. S. P. H., No. 26*, 1906, 43.

[15] Armstrong, H. E., and Ormerod, E., *Proc. Roy. Soc. London, Series B*, 1906, lxxviii, 376.

[16] de Hemptinne, A., *Z. physik. Chem.*, 1894, xiii, 561.

[17] Price, T. S., *Öfversigt K. Vetenskapsakad. förhandlingar*, 1899, lvi, 921.

[18] Dean, E. W., *Am. J. Sc.*, 1913, series 4, xxxv, 605; 1914, series 4, xxxvii, 331.

only available information on acid hydrolysis has been obtained with incompletely dissolved esters, an objection that applies particularly to many of the experiments of Morel and Terroine.[19] Data that are doubtful for this or any other reason have been omitted from the table. The provision of more satisfactory measurements of the rate of acid hydrolysis might permit an appreciable enlargement of the list.

Although most of the lipase experiments on record have been made without the addition of any buffer, and hence in acid solution, it does not necessarily follow that acid hydrolysis is the

TABLE XIV

Comparison of Orders of Velocity of Hydrolysis by Lipase and by Acid.

Lipase	Acid
Methyl butyrate>propionate>acetate.[14]	Methyl propionate>acetate>butyrate.[16,17]
Ethyl butyrate>propionate>acetate.[13,14,15]	Ethyl propionate>acetate>butyrate.[16,17,18]
Ethyl>methyl acetate.[14]	Ethyl=methyl acetate.[16,17]
Ethyl>methyl propionate.[14]	Ethyl=methyl propionate.[16]
Ethyl>methyl butyrate.[14]	Ethyl=methyl butyrate.[16]
Propyl>ethyl acetate.[19]	Propyl=ethyl acetate.[16] *
Triacetin>diacetin.†	Triacetin=diacetin.‡

* Taylor, H. S., *Medd. K. Vetenskapsakad. Nobelinst.*, 1913, ii, No. 34.

† Loevenhart, A. S., and Souder, C. B., *J. Biol. Chem.*, 1906–07, ii, 415. Loevenhart, A. S., *J. Biol. Chem.*, 1906-07, ii, 427.

‡ Yamasaki, E., *J. Am. Chem. Soc.*, 1920, xlii, 1455. The same is true of alkaline hydrolysis (Meyer, J., *Z. physik. Chem.*, 1909, lxvii, 257. Smith, L., *Z. physik. Chem.*, 1922, cii, 54.)

proper basis for comparison with lipase. Recent work[20,21] from von Euler's laboratory indicates that the dividing line between acid and alkaline hydrolysis, in the case of esters, is not at the neutral point, but a considerable distance to the acid side of it. For example, methyl and ethyl acetates are hydrolyzed at the same rate in strongly acid solution,[16,17] but the former much more

[19] Morel, L., and Terroine, É.-F., *J. physiol. et path. gén.*, 1912, xiv, 58.

[20] von Euler, H., and Laurin, I., *Ark. Kemi, Mineral. och Geol.*, 1919, vii, No. 30. von Euler, H., and Svanberg, O., *Z. physiol. Chem.*, 1921, cxv, 139.

[21] Karlsson, K. G., *Z. anorg. u. allg. Chem.*, 1921, cxix, 69.

rapidly in strongly alkaline solution.[22,23] As the acidity is increased, the rates for the two esters become equal, not at pH 7, but at about pH 4.[21] In the presence of lipase, ethyl acetate may be decidedly the less stable of the two[24] and, as long as the acidity does not reach the limit mentioned, lipase must then be considered to have reversed the order of stability. When the percentage of hydrolysis by lipase, and therefore the amount of acid produced, is small, it is accordingly probable that those steps in the process which do not involve the enzyme itself are the same that occur when the ester is hydrolyzed by alkali.

Partly because the hydrolysis of esters by alkali has been more extensively studied than acid hydrolysis, the number of known cases in which lipase modifies the order of stability to *alkali* is considerably greater (Table XV). Furthermore, it happens that the rate of hydrolysis by alkali in nearly every series that has been studied diminishes regularly as the molecular weight rises, and wherever lipase has been shown definitely to modify the order, it actually reverses it.

The following general conclusion may then be drawn. In any of these series of homologous esters, as the molecular weight rises the rate of hydrolysis by acid or alkali tends to diminish (although, in the particular cases accepted for inclusion in Table XIV, changing the alcohol radicle has no significant effect on the rate of acid hydrolysis). Within certain limits, lipase tends to modify, and usually to reverse, this order, making the rate of hydrolysis parallel with the molecular weight.

Reactions in the Living Animal.

Information in the literature on the relative stability of surface active homologs in the body is confined, as far as I have been able to learn, to observations on alcohols, fatty acids, and ketones.

[22] Reicher, L. T., *Ann. Chem.*, 1885, ccxxviii, 257; 1886, ccxxxii, 103.

[23] Trautz, M., and Volkmann, K. T., *Z. physik. Chem.*, 1908, lxiv, 53.

[24] Under some conditions there is apparently no marked difference between the rates of hydrolysis of these two esters by lipase (Morel and Terroine,[19] and Falk[25]). Since the order of stability appears to be the resultant of two opposing factors, one or the other of these may be expected to predominate, according to the circumstances.

[25] Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 616.

The reactions involved in the oxidation of such compounds in the body are much more complex than the hydrolysis of esters or amides, and they occur in several steps, the nature of which is not definitely known. As long as that is so, there is nothing with

TABLE XV.

Cases in Which Lipase Reverses the Order of Alkaline Hydrolysis.

Order of velocity of lipase hydrolysis.	Observer.	
	Lipase.	Alkali
Methyl butyrate>propionate>acetate.	Kastle.[14]	Trautz and Volkmann.[23]
Ethyl butyrate>propionate>acetate.	Kastle and Loevenhart;[13] Kastle; Armstrong and Ormerod;[15] Morel and Terroine;[19] Falk.[25]	Reicher;[22] Trautz and Volkmann; Dean.[18]
Diethyl succinate>malonate.	Armstrong and Ormerod; Morel and Terroine; Christman and Lewis.*	Skrabal and Singer.†
Diethyl glutarate>succinate.	Morel and Terroine.	" " "
Diethyl suberate>malonate.	" " "	" " "
Ethyl>methyl acetate.[24]	Kastle.	Reicher; Trautz and Volkmann.
Ethyl>methyl propionate.	"	Trautz and Volkmann.
Ethyl>methyl butyrate.	"	" " "
Propyl>ethyl acetate.	Morel and Terroine.	Reicher; Trautz and Volkmann.
Isoamyl>ethyl acetate.	Armstrong and Ormerod.	" " "

* The experiments of Christman and Lewis with these two esters were done under apparently uniform conditions with a stable preparation of hepatic lipase, but the authors do not say whether the conditions were strictly comparable (Christman, A. A., and Lewis, H. B., *J. Biol. Chem.*, 1921, xlvii, 495).

† Lipase removes only one ethyl group (Christman and Lewis). Skrabal and Singer were the first to make a separate investigation of the first step in the reaction with alkali (Skrabal, A., and Singer, E., *Monatsh. Chem.*, 1920, xli, 339).

which the enzyme reaction may be compared and, even if the rate of hydrolysis in the body is parallel with the molecular weight, it is impossible to say whether the enzyme really changes the order of stability. Some of the work is also subject to difficulties of interpretation on other grounds, and in some cases the number of homologs examined on a quantitative basis is very small. For these reasons, only a brief mention of the experimental facts will be undertaken here.

Alcohols.—That ethyl alcohol is more rapidly oxidized than methyl alcohol has been found repeatedly.[26] The particular objection to this case is that it is limited to two members of the series.

Fatty Acids.—Schotten[27] administered the sodium salts of the saturated fatty acids (from formic to caproic) to dogs, and recovered from the urine about 25 per cent of the formic acid, 10 per cent of the acetic acid, and very little (no figures are given) of the higher members (for further literature see Dakin[28]). Increasing rate of oxidation as the molecular weight rises is only one of the possible explanations for these results.[28]

Ketones.—The most complete and definite set of observations is that of Schwarz[29] on homologous ketones (acetone, methylethyl ketone, methylpropyl ketone, and diethyl ketone).[30] Except in the case of acetone, he made no separate analyses of the urine, but determined only the total excretion; *i.e.*, by both urine and expired air. According to the average results of those experiments in which the doses were roughly equivalent, he recovered 59, 32, 24, and 9 per cent, respectively, of the amount given. As he and others[31] have found, the excretion of acetone takes place for the most part through the lungs. If that is true also of the homologs

[26] For the literature see Nicloux, M., and Placet, A., *J. physiol. et path. gén.*, 1912, xiv, 916; Pohl, J., *Arch. exp. Path. u. Pharmakol.*, 1918, lxxxiii, 204.

[27] Schotten, C., *Z. physiol. Chem.*, 1882–83, vii, 375.

[28] Dakin, H. D., Oxidations and reductions in the animal body, London, 2nd edition, 1922, 26.

[29] Schwarz, L., *Arch. exp. Path. u. Pharmakol.*, 1898, xl, 168.

[30] Since the last two are isomers, not more than three of these ketones belong to any one homologous series.

[31] Widmark, E. M. P., *Acta med. Scand.*, 1919–20, lii, 87; *Biochem. J.*, 1920, xiv, 364, 379. Briggs, A. P., and Shaffer, P. A., *J. Biol. Chem.*, 1921, xlviii, 413.

of acetone, Schwarz's experiments indicate that the higher ketones are more rapidly oxidized, for the partial pressure of surface active homologs over their (equivalent) aqueous solutions increases with the molecular weight,[32] and the higher members should therefore be more readily excreted.

Taken as a whole, these observations are at least suggestive of the possibility that the reactivity of surface active homologs in the animal body within certain limits tends to increase, rather than to decrease, as the molecular weight rises, hence differing from the general rule for reactions outside the body. But chiefly because these oxidation reactions cannot at present be compared *in vivo* and *in vitro*, the evidence which they furnish is not by itself very definite. In conjunction with the other cases discussed, *viz.*, ester hydrolysis by lipase *in vitro* and amide hydrolysis in the living animal, it is entitled to some consideration.

Mechanical Adsorption in Enzyme Action.

The one property which follows the order of molecular weight in homologous series, and which is at the same time capable of furnishing a possible explanation of the facts recorded above, is mechanical adsorbability.[33] The extent to which surface active homologs are adsorbed at the air surface of an aqueous solution increases, according to the well known Gibbs theorem, with the surface activity, and therefore also with the molecular weight.[34] From the standpoint of the relation of surface activity to heterogeneous catalysis in aqueous media, and hence to enzyme

[32] See, *e.g.*, Worley, R. P., *J. Chem. Soc.*, 1914, cv, 273.

[33] Only one of the four amides used in the experiments reported in this paper (acetamide) has previously been shown to be adsorbed by charcoal.[37] Accordingly, a 0.2 M solution of each of the four was shaken (at 27°C.) with Merck's blood charcoal, in the proportion of 1 gm. of charcoal for each 10 cc. of solution. The charcoal was filtered off, and the concentration of amide in each filtrate determined by analyzing for total nitrogen, with the following results: acetamide 0.1106 M, propionamide 0.0884 M, *n*-butyramide 0.0544 M, and *n*-valeramide 0.0242 M. The adsorption hence increases with the number of carbon atoms, as in other series.

[34] Duclaux, E., *Ann. chim. et phys.*, 1878, series 5, xiii, 76. Traube, I., *Ann. Chem.*, 1891, cclxv, 27; *Arch. ges. Physiol.*, 1904, cv, 541. Forch, C., *Ann. Physik. u. Chem.*, 1899, series 2, lxviii, 801. Neidle, M., *J. Am. Chem. Soc.*, 1915, xxxvii, 513.

action, the important question is to what extent this kind of adsorption may be expected to take place at the surfaces of particles suspended in water. While there is no sound theoretical basis, analogous to the Gibbs theorem, relating surface activity and adsorption by suspended particles,[35] yet the fact remains that any solid which adsorbs such compounds to any demonstrable degree shows greater adsorption, within any one homologous series, as the length of the carbon chain increases.[36,37,38]

Michaelis and Rona,[38] in a series of recent papers, are inclined to consider that the adsorption of surface active organic compounds by charcoal is due to the unsaturated nature of elementary carbon, because charcoal, gram for gram, is a much more effective adsorbent for such compounds than any other solid that has been examined. They, themselves, however, find that considerable adsorption occurs with such widely different substances as talc and sulfur, and some with cellulose, and all these were shown to adsorb homologs in the order of their molecular weights. Michaelis and Rona's assumptions with regard to the surface areas of their various adsorbents are unproved and, since the area (or apparent area[39]) of the charcoal surface is extraordinarily large,[40] it remains an open question whether charcoal is really as unique as they suppose it to be.

[35] Bancroft, W. D., *J. Franklin Inst.*, 1918, clxxxv, 199; Applied colloid chemistry; general theory, New York, 1921, 135.

[36] Walker, J., and Appleyard, J. R., *J. Chem. Soc.*, 1896, lxix, 1334. Freundlich, H., *Z. physik. Chem.*, 1907, lvii, 385. Traube, I., *Verh. deutsch. physik. Ges.*, 1908, x, 880. Michaelis, L., and Rona, P., *Biochem. Z.*, 1909, xv, 196. Rona, P., and von Tóth, K., *Biochem. Z.*, 1914, lxiv, 288. Somogyi, R., *Int. Z. physik. chem. Biol.*, 1915–16, ii, 412. Berczeller, L., and Hetényi, S., *Biochem. Z.*, 1917, lxxxiv, 137. Berczeller, L., *Kolloid-Z.*, 1918, xxiii, 31. Wiegner, G., Magasanik, J., and Virtanen, A. J., *Kolloid-Z.*, 1921, xxviii, 51. *Cf.* also Brown, A. J., and Tinker, F., *Proc. Roy. Soc. London, Series B*, 1915–17, lxxxix, 373.

[37] Warburg, O., *Biochem. Z.*, 1921, cxix, 134.

[38] Michaelis, L., and Rona, P., *Biochem. Z.*, 1919, xcvii, 57; *Kolloid-Z.* 1919, xxv, 225; *Biochem. Z.*, 1920, cii, 268. Rona, P., and Michaelis, L., *Biochem. Z.*, 1920, ciii,19.

[39] See Langmuir, I., *J. Am. Chem. Soc.*, 1916, xxxviii, 2286.

[40] Lowry, H. H., and Hulett, G. A., *J. Am. Chem. Soc.*, 1920, xlii, 1393. Lamb, A. B., Wilson, R. E., and Chaney, N. K., *J. Ind. and Eng. Chem.*, 1919, xi, 420.

Michaelis and Rona's hypothesis, therefore, does not dispose of the possibility of mechanical adsorption by enzymes. As for the experimental evidence for such a process, it is necessarily of an indirect nature, consisting in the first place of the phenomenon of enzyme inhibition by soap, saponin, and other substances capable of forming viscous surface films, which presumably prevent contact between enzyme and substrate. Further evidence is furnished by the inhibitory effect of homologs, which has been shown on various occasions to run parallel with the molecular weight, just as adsorption does. This was first demonstrated by Linossier[41] for the inhibition of pepsin, rennin, trypsin, and invertase by homologous alcohols, and Meyerhof[42] several years later made similar observations on the inhibition of invertase by alcohols, ketones, urethanes, and substituted ureas. Displacement of the substrate from the enzyme surface would be expected to follow the same order, and these facts may be explained in that way, as Meyerhof suggests. Similar results have been reported by Chapman,[43] Onodera,[44] and others (mostly under more complicated circumstances).[45] In some cases, but not in all, the inhibition is accompanied by flocculation of the enzyme.[46] (While flocculation may be partly due to adsorption, another possible cause is a change in the dielectric constant of the medium, and the situation is accordingly less clear when the physical condition of the enzyme is obviously altered.) Similar inhibitory effects by surface active substances, with the same relation to molecular weight, have been observed in catalysis by colloidal platinum[47] and by charcoal.[37, 48]

The question has been approached from another angle by Bayliss,[49] who succeeded in showing that the inhibition of urease

[41] Linossier, G., *Compt. rend. Soc. biol.*, 1899, li, 887.

[42] Meyerhof, O., *Arch. ges. Physiol.*, 1914, clvii, 251.

[43] Chapman, G. H., *Int. Z. physik. chem. Biol.*, 1914, i, 293. *Cf.* Meyerhof, O., *Int. Z. physik. chem. Biol.*, 1915–16, ii, 394.

[44] Onodera, N., *Biochem. J.*, 1915, ix, 544.

[45] See Winterstein, H., Die Narkose, Berlin, 1919, pp. 197–199.

[46] Winterstein,[45] pp. 248–258.

[47] Meyerhof, O., *Arch. ges. Physiol.*, 1914, clvii, 307.

[48] Warburg, O., *Arch. ges. Physiol.*, 1914, clv, 547. Freundlich, H., and Bjercke, A., *Z. physik. Chem.*, 1916, xci, 1.

[49] Bayliss, W. M., *Arch. néerl. physiol.*, 1917–18, ii, 621.

by amyl alcohol and by saponin becomes more pronounced as the temperature falls, as it should if adsorption is the cause of the inhibition.

All this work is concerned with the effect of surface active substances on the activity of enzymes engaged in decomposing something else. The tendency, discussed earlier in this paper, for the reactivity of substances of that character to increase with the molecular weight in the presence of biological material possibly shows the influence of mechanical adsorption when the surface active compound is itself the substrate.

At all events, the adsorption of surface active homologs increases with the length of the hydrocarbon chain, and within certain limits the rate of their decomposition by enzymes does likewise. The mechanical adsorption of substrate by enzyme is a possible explanation for this, and no other property appears to be capable of furnishing any explanation at all for this seemingly unique characteristic of biological reactions.

The same facts constitute a certain amount of evidence for the view that adsorption may be a determining factor in enzyme action purely by producing a local increase in concentration.[50] They have, of course, no bearing on the mechanism of enzyme action with substrates possessing no appreciable degree of surface activity, nor do they in any case exclude intermediate compound formation as an additional factor.

EXPERIMENTAL.

The cats (female) used in these experiments, after a preliminary fast of a few days, were kept in metabolism cages. To avoid catheterization, the bladder was emptied by compressing the abdomen about 3 hours before the end of each 24 hour period, and 100 cc. of distilled water were given by stomach tube; at the end of the period the bladder was again emptied in the same manner. The last fraction of the 24 hour urine is in this way made very dilute, and the error incurred by leaving a small amount of urine in the bladder is not significant. Since the bacterial decomposi-

[50] See Bayliss, W. M., Second report on colloid chemistry and its general and industrial applications, London, 2nd edition, 1921, 143. Denham, H. G., *Z. physik. Chem.*, 1910, lxxii, 641.

tion of amide would have been particularly disastrous here, catheterization would not have been safe. As far as may be judged by the constancy of the creatinine (or total creatinine) output, the substitute method used is quite adequate for the

TABLE XVI.

Experiment 16. Fasting cat.

Day of fast.	Volume.	Total N.	Urea + NH_3-N.	Ammonia N	Creatinine N.	Undetermined N.	Total S.	Inorganic sulfate S	Ethereal sulfate S.	Neutral S.
	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
3	108	0.452	0.390	0.018	0.0161	0.046	0.0287	0.0154	0.0055	0.0078
4	91	0.478	0.418	0.023	0.0162	0.044	0.0298			
5	124	0.462	0.398	0.020	0.0157	0.048	0.0303	0.0165	0.0056	0.0082
6}	88	0.467	0.402	0.019	0.0163	0.049	0.0330	0.0201	0.0046	0.0084
7}	88	0.467	0.402	0.019	0.0163	0.049	0.0330	0.0201	0.0046	0.0084
8	97	0.535	0.470	0.030	0.0163	0.049	0.0349	0.0237	0.0033	0.0079
9	72	0.495	0.435	0.030	0.0153	0.045	0.0356	0.0231	0.0034	0.0091

Day of fast.	Per cent of total nitrogen.				Per cent of total sulfur.			$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total sulfate S.}}$
	Urea + NH_3-N.	Ammonia N.	Creatinine N.	Undetermined N.	Inorganic sulfate S	Ethereal sulfate S	Neutral S.	
3	86.3	4.0	3.6	10.2	53.7	19.1	27.2	18.7
4	87.4	4.8	3.4	9.2				18.0
5	86.2	4.3	3.4	10.4	54.6	18.4	27.0	18.0
6}	86.1	4.1	3.5	10.5	60.9	13.7	25.4	16.2
7}	86.1	4.1	3.5	10.5	60.9	13.7	25.4	16.2
8	87.8	5.6	3.0	9.2	67.9	9.5	22.6	17.4
9	87.9	6.1	3.1	9.1	64.9	9.6	25.5	16.4

sharp separation of the urine into 24 hour periods (see control experiment, Table XVI).[51]

The amide to be injected was dissolved in from 5 to 25 cc. of water, depending upon its solubility, and the solution with its

[51] The creatine nitrogen was uniformly about 2 mg.

containing beaker sterilized with steam. When the solution had cooled to about body temperature, the injection was made with a syringe that had been sterilized in boiling water, and the beaker and syringe were then rinsed out with about 10 cc. of warm sterile water, the washings being finally injected under the skin at a different site.

All possible precautions were taken to avoid changes in the composition of the urine between collection and analysis. When, for example, the periods ended, as they usually did, at noon, the the urine that had been collected at about 9 a.m. was kept, preserved with chloroform, in the ice box. The last fraction of the 24 hour urine, collected at 12 m., was added to this, and the analyses were begun within less than 1 hour.[52] The only exceptions to this occurred when other matters interfered with collecting the urine accurately at the expiration of the 24 hours. When this happened during the fore or after period, each sample was at once preserved with chloroform and removed to the ice box, and the whole 48 hour specimen analyzed promptly at the end of the following period. Not even this liberty was taken during the excretion of amide, when the two approximately 24 hour urines were analyzed separately, and the results averaged.

These precautions were perhaps not altogether necessary. On a number of occasions, a few cubic centimeters of the urine were transferred to a separate bottle and allowed to stand (with chloroform) for 24 hours at room temperature; in no case, whether amide was present or not, was the ammonia content any higher than before. Nevertheless, any appreciable hydrolysis of amide after excretion might seriously have affected the interpretation of some of these experiments, and it was safer to take no chances. Without the added security derived from paying particular attention to this point, the increased ammonia excretion that followed the administration of amide in some instances might justly have been attributed to bacterial decomposition. Even in the alkaline

[52] Most cats at first show no inclination to urinate spontaneously (the occasional exceptions were used for other experiments). In the course of a few days some of them do so, but they conveniently select about the time of day at which they have become accustomed to having the bladder emptied by compression. Spontaneous urination in a few instances occurred at odd times in the experiments reported, but never when it could have made any difference in the result.

rabbit urines (Experiment 12, Table VI), where the conditions are more favorable for bacterial growth as well as for detecting ammoniacal decomposition, no hydrolysis occurred when the amide excretion was at its height. The urines of the last 2 days were analyzed together, and even there the figure for ammonia nitrogen cannot be more than 1 mg. too high.

Analytical Methods.

Total nitrogen was determined by the Kjeldahl-Gunning method; urea by the urease method;[53] ammonia by the Folin-Macallum colorimetric method;[54] total sulfur by the Denis[55] modification of Benedict's method; total sulfate according to Folin.[56]

Determination of Amide.—A suitable quantity of urine (enough to yield from 0.75 to 1.5 mg. of ammonia nitrogen when hydrolyzed according to the directions to be given) is transferred to a large test-tube of hard glass, and diluted with water to 4 cc. 1 cc. of concentrated hydrochloric acid (sp. gr. 1.18) is added, and the tube provided with a rubber stopper carrying a calcium chloride tube to serve as an air condenser. The contents of the tube are then heated to boiling with a micro burner, and gentle boiling is maintained for exactly 10 minutes. After being allowed to cool for 5 minutes, the mixture is aerated with 2 cc. of saturated sodium hydroxide solution (and a few drops of kerosene), exactly as in the Folin-Farmer method for total nitrogen.[57] The contents of the receiving flask are nesslerized in the usual manner, and compared in the colorimeter with a standard containing 1 mg. of ammonia nitrogen. The analysis may also be made by titration, but a second test-tube, containing a few cc. of saturated sodium hydroxide solution, must then be interposed between digestion tube and receiver to catch the hydrochloric acid vapor that comes over.[58]

[53] Fiske, C. H., *J. Biol. Chem.*, 1915, xxiii, 455.
[54] Folin, O., and Macallum, A. B., *J. Biol. Chem.*, 1912, xi, 523.
[55] Denis, W., *J. Biol. Chem.*, 1910, viii, 401.
[56] Folin, O., *J. Biol. Chem.*, 1905–06, i, 131.
[57] Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.
[58] Bock, J. C., *J. Biol. Chem.*, 1913, xiv, 295.

From the result of this analysis it is necessary to subtract (1) the preformed ammonia nitrogen, and (2) the urea correction. The average urea correction, based on a large number of analyses of urines from normal fasting cats, is 1.5 per cent of the urea content. When that correction is applied, after subtracting the preformed ammonia nitrogen, there is left a remainder amounting to ± 0.005 gm. of nitrogen per 24 hours. Since urea in pure aqueous solution is hydrolyzed to about that extent when treated as in the amide determination, it is at least the main constituent of such urines yielding ammonia on hydrolysis with acid, and there is evidently no significant quantity of easily hydrolyzable

TABLE XVII

Determination of Amides Added to Urine *

Amide	Amide N added	Urea N	Ammonia N	N by HCl hydrolysis	Amide N found †
	gm	*gm*	*gm*	*gm*	*gm*
Acetamide	0.377	0.990	0.026	0.427	0.386
	0.152	0.852	0.080	0.246	0.153
	0.008	0.852	0.080	0.105	0.012
Propionamide	0.600	0.840	0.031	0.655	0.611
	0.180	0.850	0.037	0.237	0.187
	0.060	0.850	0.037	0.111	0.061
n-Butyramide	0.600	0.838	0.031	0.648	0.604
	0.179	0.850	0.037	0.236	0.186
	0.060	0.850	0.037	0.113	0.063
n-Valeramide	0.020	0.850	0.037	0.074	0.024

* 24 hour urines from fasting cats.

† (N by HCl hydrolysis) – (ammonia N) – (0.015 × urea N).

amide present. Since the average urea correction has varied slightly with different animals, the correction actually used has been determined separately for each one by analyzing the urines collected during the preliminary period. When that is done, the accuracy of the amide method is probably slightly greater than indicated in Table XVII, which shows the nature of the results obtained using a flat correction of 1.5 per cent. The error in the analysis of pure amide solutions need not exceed 1 per cent; with urine (under the conditions of the experiments reported in this paper) an additional error of 0.005 gm. of amide nitrogen per day may be expected. Depending upon whether the amide content is

large or small, this method is from five to ten times as accurate as the best results that I have been able to get with the distillation method on the same kind of material.

None of the four amides used has been found to have any effect on the ammonia determination. This merely confirms the work of others[59] who have detected no decomposition of acetamide (the least stable of the four in alkaline solution) during aeration with sodium carbonate for much longer periods than I have used.

The urea determination likewise is not affected by the presence of these amides. They are not decomposed by urease,[60] and even if they should have any inhibiting influence on the enzyme (as some surface active compounds do), no harm would be done in the analysis, for the duplicate urea determinations were invariably run with slightly different amounts of the enzyme solution. In other words, the activity of the urease was controlled in every analysis.

Materials.

The acetamide (Kahlbaum), propionamide (Eimer and Amend), and *n*-butyramide (partly obtained from Eimer and Amend, and partly prepared from methyl butyrate by Meyer's method[61]) were all recrystallized from benzene until the melting point was within 1° of the highest figure recorded, and the nitrogen content within 0.2 per cent of the theoretical figure.

n-Valeramide.—This amide has usually been made from *n*-valeric acid, the most convenient source of which is valeronitrile.[62,63,64] In order to save several steps, the amide used in this work was prepared directly from the nitrile.[65] The details of one such preparation follow.

[59] Potter, R. S., and Snyder, R. S., *J. Ind. and Eng. Chem.*, 1915, vii, 220. Davisson, B. S., *J. Ind. and Eng. Chem.*, 1918, x, 600.

[60] *Cf.* Labberte, K. R., *Pharm. Weekbl.*, 1915, lii, 1428; abstracted in *Chem. Abstr.*, 1916, x, 1359.

[61] Meyer, H., *Monatsh. Chem.*, 1906, xxvii, 31.

[62] Lieben, A., and Rossi, A., *Ann. Chem.*, 1871, clix, 58.

[63] Gartenmeister, R., *Ann. Chem.*, 1886, ccxxxiii, 272.

[64] Adams, R., and Marvel, C. S., *J. Am. Chem. Soc.*, 1920, xlii, 311.

[65] *Cf.* Marckwald, W., and Nolda, E., *Ber. chem. Ges.*, 1909, xlii, 1583.

25 gm. of *n*-valeronitrile[62,66,67] were mixed with 125 cc. of hydrochloric acid (sp. gr. 1.18) and let stand for 48 hours. The solution was then evaporated *in vacuo* at about 40° until crystals (valeramide hydrochloride) began to appear, and the residue transferred to a beaker (with about 200 cc. of water) and neutralized to methyl red with sodium carbonate. (About 3 per cent of the amide is hydrolyzed during the evaporation.) The solution was heated on the steam bath, the reaction being adjusted to the neutral point from time to time until all the carbon dioxide had been driven off, and evaporated to a volume of 175 cc. On cooling, the amide separated in plates, weighing 18.0 gm. after being dried *in vacuo* over sulfuric acid. The filtrate, evaporated to 65 cc. and allowed to cool, gave a second crop of 6.2 gm., and 0.5 gm. more was obtained by extraction with ether. The total yield was 24.7 gm., or 81 per cent of the theoretical.

The product so obtained contains a little sodium chloride. This may be removed by dissolving the amide in 10 parts of dry alcohol-ether (1:4) and filtering. On evaporating the filtrate to dryness, and recrystallizing from 2 parts of water, a pure product melting at 106° (corrected)[68] results.

0.1831 gm. (Kjeldahl) required 18.06 cc. 0.1 N HCl, equivalent to 0.02531 gm. of nitrogen.

$C_5H_{11}ON$.	Calculated.	N 13 86.
	Found.	" 13 83.

Hydrolysis of Butyramide and Valeramide by Acid and Alkali.

The hydrolyses were conducted in sealed tubes of Jena glass containing 5 cc. of approximately 0.2 M amide and 5 cc. of 0.2 N hydrochloric acid or sodium hydroxide. The tubes were heated in an atmosphere of steam, and the results corrected to 100.0°C. by means of barometer readings, and the temperature coefficients found by Crocker[11] (the influence of temperature is nearly the same for different members of the series). The maximum possible error in *k* due to temperature lag at the beginning of the reaction

[66] Lieben, A., and Rossi, A., *Ann. Chem.*, 1871, clviii, 171.

[67] The preparation of this substance has recently been improved by Adams and Marvel.[64]

[68] Robertson, P. W., *J. Chem. Soc.*, 1919, cxv, 1210.

was found to be 1.5 per cent. Since the time required for cooling at the end largely compensates for this, the actual error must have been much less, and it has consequently been disregarded.

The reaction was stopped by cooling the tube in running water, the tube opened, and ammonia determined in an aliquot portion of the solution by aeration and titration (to methyl red). The results are given in Table XVIII.

TABLE XVIII.

Hydrolysis of Amides by Acid and Alkali.

Amide.	Catalyst.	Barometric pressure.	a	b	Time (t).	x	k	k at 100 0°C.
		mm Hg	N	M	*min.*	N		
n-Butyramide.....	HCl	765	0.1000	0.0990	75	0.0633	0.235	0.231
		765	0.1000	0 0990	85	0.0661	0 234	0 230
n-Valeramide.	HCl	767	0.1000	0.0968	76	0.0634	0 242	0 237
		760	0.1000	0.0968	75	0 0630	0.241	0.241
n-Butyramide ...	NaOH	765	0.1000	0 0990	75	0.0490	0.130	0.129
		762	0.1000	0.0990	75	0.0488	0.129	0.128
n-Valeramide.	NaOH	762	0.1000	0.0968	75	0.0429	0.105	0.105
		759	0.1000	0.0968	75	0.0428	0.104	0.104

a = initial concentration of hydrochloric acid or sodium hydroxide.
b = initial concentration of amide.
x = concentration of ammonia produced in t minutes.

$$k = \frac{1}{t\,(a-b)} \ln \frac{b(a-x)}{a(b-x)}.$$

SUMMARY.

When equivalent doses of acetamide, propionamide, n-butyramide, and n-valeramide are given subcutaneously to fasting cats, the amount of unchanged amide excreted in the urine diminishes progressively as the number of carbon atoms increases. From this and other evidence, it is concluded that the rate of hydrolysis of these amides in the body increases with the length of the paraffin chain.

The order of stability to ordinary hydrolyzing agents in the test-tube is not the same. When hydrolyzed by acid, propionamide is slightly less stable than acetamide, but butyramide and valeramide are much more stable than either of the others. In alkaline solution the order is just the reverse of that found in the body; *i.e.*, the stability increases with the molecular weight.

Consideration of the literature on ester hydrolysis by lipase and on certain reactions in the animal body (the oxidation of alcohols, fatty acids, and ketones) shows that the rate of decomposition of surface active homologs by enzymes tends to increase with the number of carbon atoms, contrary to the general rule with ordinary reagents in true solution.

Since the adsorbability of surface active homologs increases with the length of the carbon chain, it is suggested that the tendency for their reactivity to enzymes to follow the same order is due to mechanical adsorption of the substrate by the enzyme.

EPIGLUCOSAMINE.

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In recent years two nitrogenous hexoses have been prepared which, on the basis of their preparation, may be either isomeric or identical. One was prepared by Levene[1] in 1919 and the other by Fischer, Bergmann, and Schotte[2] in 1920. The first was named epichitosamine. The starting material for its preparation was chitosaminic acid which was converted into the epimeric acid by the action of pyridine. On the reduction of the lactone of epichitosaminic acid, epichitosamine was obtained. Fischer, Bergmann, and Schotte named their substance epiglucosamine. In this publication it will be referred to under this name. Epiglucosamine was prepared in the form of its methylglucoside by the action of ammonia on methylglucoside-2-chlorohydrin. Fischer, Bergmann, and Schotte point out the uncertainty which exists in regard to the position of the amino group in their substance. Unfortunately, they found it difficult to remove the methyl group from the 1-methylepiglucosamine.

Since epichitosamine may be regarded as a true epiglucosamine, a comparison of the two substances should have revealed the structure of the epiglucosamine of Fischer, Bergmann, and Schotte. Hence the plan of this work was to prepare the methylglucoside of epichitosamine and to compare its properties with those of 1-methylepiglucosamine. Unfortunately, the preparation of epichitosamine is a time-consuming process and before this part of the work was concluded, the work on epiglucosamine revealed some important details of its structure.

The monomethylepiglucosamine was prepared directly from triacetylmethylglucoside-2-chlorohydrin or bromohydrin. The

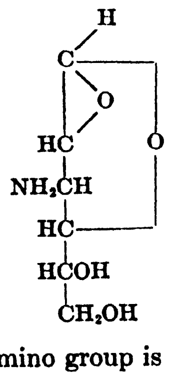
[1] Levene, P. A., *J. Biol. Chem.*, 1919, xxxix, 69.

[2] Fischer, E., Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, 1920, liii, 509.

substance obtained in this manner is the acetic acid salt of the base $C_7H_{15}O_6N$. It was found that the difficulty in obtaining the free sugar was occasioned not by the great resistance of the glucoside towards acid hydrolysis, but by the fact that the free sugar in the presence of fairly concentrated mineral acids was rapidly changing into an inner glucoside (anhydroepiglucosamine). On hydrolysis with very dilute acid it was possible to obtain an equilibrium containing approximately 60 per cent of the free sugar. In this condition the free sugar could be readily transformed into an osazone. The osazone differed in its solubility greatly from the osazones of the other hexoses. It contained 5 atoms of nitrogen in its molecule. Thus it became evident that epiglucosamine has its amino group in position 3. As pointed out by Fischer, Bergmann, and Schotte, prior to the formation of the amino sugar an ethylenoxide ring may be established between carbon atoms 2 and 3. It is then not certain whether on further action of ammonia the position of the hydroxyl on carbon atom 2 is changed to that in mannose or remains as it was in glucose. Hence epiglucosamine may have one of the four following configurations (the configuration of only the four middle carbon atoms is given):

OH	H	H	OH	H	OH	OH	H
H	NH_2	H	NH_2	NH_2	H	NH_2	H
H	OH	H	OH	H	OH	H	OH
H	OH	H	OH	H	OH	H	OH

The structure of the anhydro sugar is



The allocation of the amino group is arbitrary.

EXPERIMENTAL.

Triacetylmethylchlorohydrin was prepared according to the directions of Fischer, Bergmann, and Schotte. This material was then taken up in 10 volumes of concentrated ammonia water and heated in an autoclave at 100°C. for 15 hours. The product was concentrated to dryness, taken up in boiling methyl alcohol, and allowed to crystallize. The average yield was 10 gm. of methylepiglucosamine acetate from 20.0 gm. of triacetylmethylchlorohydrin. The yield from the corresponding bromohydrin was not equally satisfactory. For analysis, the substance was recrystallized from methyl alcohol. The substance turned brown at 210°C. and melted at 214°C. (corrected).

0.1007 gm. substance: 0.1576 gm. CO_2 and 0.0700 gm. H_2O.
0.0994 " " required for neutralization 4.0 cc. 0.1 N acid.
0.0200 " " : (Van Slyke) 2.04 cc. N_2, t = 24°C., P = 761 mm.

$C_7H_{15}O_5N$. Calculated.	C 39.80,	H 7.94,	N 5.89,	Amino N 5.89.
Found.	" 42.67,	" 7.77,	" 5.63,	" " 5.70.

The optical rotation of the substance in 2.5 per cent hydrochloric acid was,

$$[\alpha]_D^{20} = \frac{-1.30° \times 100}{1 \times 1} = -130°$$

In order to prove that the substance was not a monoacetyl derivative but a salt of acetic acid, it was converted into the hydrochloride in the following way: 1.0 gm. of the substance was dissolved in hot methyl alcohol, filtered, and allowed to cool. To this solution a slight excess of absolute alcohol containing hydrochloric acid was added and to the resulting solution ether was added to a slight opalescence. The substance soon crystallized in long needles.

The substance analyzed as follows:

0.1044 gm. substance: 0.1402 gm. CO_2 and 0.0700 gm. H_2O.
0.0994 " " required for neutralization 4.40 cc. 0.1 N acid.
0.0994 " " : (Volhard) 4.35 cc. 0.1 N silver nitrate.

$C_7H_{15}O_5N \cdot HCl$. Calculated.	C 36.58,	H 7.02,	N 6.10,	Cl 15.44.
Found.	" 36.62,	" 7.50,	" 6.19,	" 15.51.

The optical rotation of the substance in 2.5 per cent hydrochloric acid was,

$$[\alpha]_D^{21} = \frac{-1.38° \times 100}{1 \times 1} = -138°$$

Hydrolysis of the 1-Methylepiglucosamine.

It was attempted to follow the progress of hydrolysis by the changes in optical rotation. If epiglucosamine were identical with epichitosamine, the ultimate rotation should be $[\alpha]_D^{21} = -4.7°$.

The changes in optical rotation in the course of hydrolysis varied with the concentration of the acid. When the concentration of the acid was very weak, there was no noticeable change in rotation in spite of the fact that the solution reduced Fehling's solution, which it failed to do prior to hydrolysis. When the concentration of the acid was slightly increased, the rotation dropped continually, but the minimum specific rotation was ten times as high as that of epichondrosamine. On hydrolysis with stronger acid there was a continuous increase in optical rotation and finally when the hydrolysis was accomplished at room temperature with very concentrated acid, a marked drop in rotation was noted which was followed by a rise beyond the original magnitude.

TABLE I.

Time.	Per cent HCl.				
	0 25	0.5	2	5	40
hrs.					
0	−1.33°	−1.32°	−1.30°	−0.22°	−4.00°
1	−1.32°	−1.32°	−0.43°	−0.50°	−1.67°
2	−1.32°	−1.32°	−0.38°	−0.65°	−3.60°
3	−1.32°	−1.32°	−0.39°		
6		−0.79°		−0.60°	
7	−1.31°				−4.58°
8	−1.26°	−0.69°			
21		−0.69°			−4.61°

Table I shows the changes in rotation with the variation in the concentration of the acid.

On hydrolysis with 0.5 per cent hydrochloric acid the equilibrium in optical rotation and in reducing power was reached at the same time, as seen from Table II.

When the product of hydrolysis, regardless of the concentration of the hydrogen chloride, was reduced in volume under diminished

pressure, a product crystallized out which did not reduce Fehling's solution, but which again acquired this property on hydrolysis. On the basis of this peculiarity as well as on the basis of the changes in the optical rotation it was concluded that a double change took place in the substance. The first was a change of the methylglucoside into the free sugar and the second of the free sugar into the inner glucoside. The second reaction is reversible.

1-Methylepiglucosamine→epiglucosamine⇄anhydroglucosamine.

The substance began to change color at 190°C. and melted with decomposition at 216° (corrected).

TABLE II.

Change in Reduction of a 1 Per Cent Solution of the 1-Methylepiglucosamine on Hydrolysis with 0.5 Per Cent HCl Compared with the Change of Optical Rotation.

Time.	Optical rotation.	Sugar in 100 cc as glucose.
hrs.		*mg.*
0	−1.32°	0
1	−1 32°	0.200
2	−1.32°	0.320
3	−1.32°	0.390
4		0.470
5		0.510
6	−0.79°	0.550
7		0.580
8	−0 69°	0.610

For analysis the substance was dried under diminished pressure at the temperature of chloroform vapors.

0.1063 gm. substance: 0.1416 gm. CO_2 and 0.0588 gm. H_2O.
0.0984 " " required for neutralization 4.95 cc. 0.1 N acid.
0.0994 " " " (Volhard) 4.88 cc. 0.1 N silver nitrate.
0.0197 " " : (Van Slyke) 2.05 cc. N_2, $t = 22°C.$, $P = 746.7$ mm.
$C_6H_{11}O_4N \cdot HCl$.

Calculated.	C 36.44,	H 6.12,	N 7.09,	Cl 17.95,	Amino N 7.09.
Found.	" 36.32,	" 6.18,	" 7.04,	" 17.40,	" " 11.51.

The abnormality of the amino nitrogen estimation by the Van Slyke method is peculiar. It was confirmed on several samples and the analysis was carried out independently by two persons.

It was found that 3-aminoheptonic acids also gave too high values for amino nitrogen in the Van Slyke apparatus.

The optical rotation of the substance in 2.5 per cent hydrochloric acid was as follows:

$$[\alpha]_D^{20} = \frac{-1.72° \times 100}{1 \times 1} = -172°$$

Phenylosazone of Epiglucosamine.

5.0 gm. of the methylglucoside were dissolved in 225 cc. of 0.5 per cent hydrogen chloride and heated with return condenser over a free flame for 8 hours. The solution remained colorless. To the warm solution 6 gm. of sodium acetate were added, together with a solution of 6 gm. of phenylhydrazine in 10 cc. of glacial acetic acid. This solution was then boiled with return condenser for 3 hours. Only a small amount of tarry material formed and this was removed by filtration. On standing for about 2 hours in a refrigerator the osazone settled out. When the osazone first begins to crystallize, it has the appearance of very small needles, on standing it turns into microscopic ball-shaped aggregates. The osazone was filtered off, transferred into glacial acetic acid, and a large excess of ether was added. After standing a short while the osazone was again filtered off. The yield of the dry material was about 0.80 to 0.90 gm. For further purification, the osazone was suspended in 35 cc. of 90 per cent methyl alcohol, boiled on the water bath, and pyridine was gradually added until the solution was completed. Hot water was then added until the solution turned opalescent. On cooling, the osazone settles out in long needles, partly in the form of rosettes. The color of the osazone is lemon-yellow. The substance is much more insoluble in organic solvents than the other phenylosazones. Thus, in Neuberg's solution of 4 parts of pyridine and 6 parts of ethyl alcohol, the osazone is insoluble even on boiling. The substance is very soluble in warm glacial acetic acid and from this solution it can be precipitated by means of ether as a bright lemon-yellow amorphous powder. The crystalline substance melts sharply at 207°C. (corrected). It analyzed as follows:

0.1014 gm. substance: 0.2252 gm. CO_2 and 0.0594 gm. H_2O.
0.1000 " " : 17.0 cc. N_2, $t = 21°C.$, $P = 767$ mm.
$C_{18}H_{22}N_5O_3$. (Mol. wt. 356.23) Calculated. C 60.63, H 6.23, N 19.66.
Found. " 60.56, " 6.55, " 19.92.

For the optical rotation the substance was dissolved in a solution of 4 parts of pyridine and 6 parts of 50 per cent (by volume) methyl alcohol. It was necessary to beat the solvent in order to bring about the solution.

Initial.

$$[\alpha]_D^{20} = \frac{-0.41° \times 100}{1 \times 1} = -41°$$

For comparison the rotation of the glucosazone was measured under the same conditions. It was found

Initial. Equilibrium.

$$[\alpha]_D^{20} = \frac{-0.57° \times 100}{1 \times 1} = -57° \qquad [\alpha]_D^{20} = \frac{-41° \times 100}{1 \times 1} = -41°$$

Thus the rotation of osazones in the new solvent is practically the same as in the solvent proposed by Neuberg.

THE HYDROGEN ION CONCENTRATION OF THE BLOOD IN CARCINOMA.

I. FROM THE COLORIMETRIC DETERMINATION OF THE BLOOD DIALYSATE.

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Several investigators have reported a difference in reaction between the blood of patients with malignant tumors and the blood of normal individuals. Moore, Alexander, Kelly, and Roaf (1906) noted a marked decrease in the total acidity of the gastric juice in cancer. The free hydrochloric acid was negative in two-thirds of the cases, regardless of the situation of the tumor. These results were confirmed by Palmer (1906). This led Moore and Wilson (1906) to titrate the serum and inorganic salts of the serum ash against standard H_2SO_4, and they found that both the serum and the serum ash of cancer patients required slightly more acid to neutralize them to a definite end-point than those of normal persons or those of cases with diseases other than cancer. Watson (1909) extended the work of Moore and Wilson with practically the same results. Menten (1917) determined the hydrogen ion concentration of the serum and the whole blood in normals, cancer cases, and other diseases by the Michaelis gas chain method. In general a greater alkalinity was found in the serum of cancer patients, but this difference did not appear in the whole blood. The reaction of the whole blood seemed to vary with the barometric pressure. As no account was taken of the loss of carbon dioxide from the blood the hydrogen ion concentration was lowered appreciably in all cases, and thus a variable factor was introduced, the value of which is difficult to estimate. All these results, however, indicate in different ways a more alkaline

reaction of the blood in cancer. Therefore, a study of the actual hydrogen ion concentration of the blood was undertaken, using the more recently developed methods and avoiding as far as known any essential changes in the blood after it was drawn. The value of a test which would give the patient with internal cancer an early diagnosis and thus the same advantage of early treatment as the one with an external cancer has often been emphasized. Toward this end the indicator method was chosen because of its adaptability as a clinical test. The results, however, do not show a sufficient difference in the early cases to warrant much diagnostic application.

Method.

The colorimetric method of determining the hydrogen ion concentration was applied to blood by Levy, Rowntree, and Marriott (1915). They dialyzed the blood in collodion sacs against neutral physiological salt solution, but did not prevent the loss of carbon dioxide from the blood or the dialysate. Scott (1917) and Dale and Evans (1920) have used essentially the same method, modified to minimize the exchange of gases between the blood and the atmosphere. A simplified combination of the two methods was employed in this experimental work. One ounce of "pyroxylin" was dissolved in 500 cc. of ether and ethyl alcohol mixed in equal volumes. The sacs were made in small Pyrex test-tubes (inside diameter 8 mm. and about 60 mm. long). These test-tubes were filled with collodion and emptied immediately, three times in succession, then inverted and drained for 15 minutes. They were then rinsed well with distilled water, the sacs were removed from the tubes and rinsed again on the outside and inside, and left in saline solution until used. Dialysis was carried out in Pyrex test-tubes (inside measurements 10 × 100 mm.) against 3 cc. of neutral 0.8 per cent NaCl.

The blood was drawn from the median vein into a glass syringe without exposure to the air and immediately discharged through the needle into a tube containing approximately 0.2 per cent crystalline potassium oxalate under a layer of pure petrolatum oil. The collodion sac was filled with petrolatum oil and placed in the dialyzing test-tube containing 3 cc. of saline solution. 1.5 to 2 cc. of blood were pipetted into the sac under the oil, forcing

the oil out above it in a layer over the whole tube and completely excluding aeration of the dialysate or blood.

The viscosity of the oil holds the sac suspended in the saline solution (Fig. 1). After dialyzing for 15 minutes the sac was removed with forceps, still leaving a sufficient layer of oil over the dialysate, and 0.2 cc. of a 0.02 per cent solution of cresol red

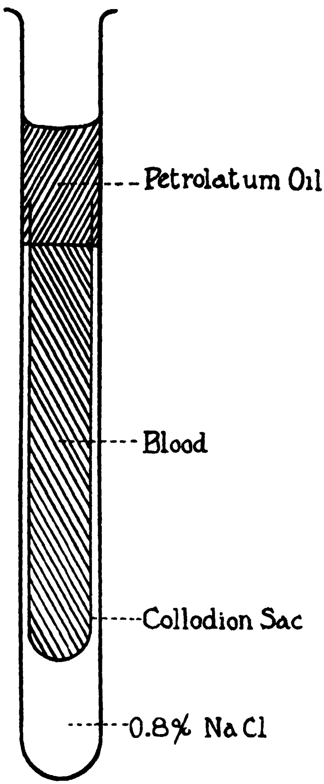

FIG. 1. The dialysis of whole blood under oil.

was added. The pH was read by comparison with standard buffer solutions which were covered by an equal layer of oil. After a comparison of the three indicators, neutral red, phenol red (phenolsulfonephthalein), and cresol red (*o*-cresolsulfonephthalein (Clark, 1920)), it was found that cresol red gave the most distinct color changes between pH 7.0 and 7.8. The KH_2PO_4—

NaOH buffer mixtures of Clark and Lubs (Clark, 1920) were used for the standard comparisons. The reagents were of highest purity and recrystallized several times. The standards varied by pH 0.10 which allowed interpolation to the second decimal.

The blood was taken between 2 and 4 hours after a meal to avoid any effect of HCl secretion on the reaction. A rest of 15 to 20 minutes was required of all normal subjects and patients not confined to bed, as Christiansen, Douglas, and Haldane (1914) have shown that exercise decreases the height of the blood CO_2 absorption curve.

Correction Factors for Temperature.

The dialysis of the blood in these experiments was carried on at room temperature which ranged between 20 and 30°C. Clark's standard phosphate solutions are prepared to give the designated pH at a temperature of 20°C. When the comparison with the dialysate is made at any temperature other than 20°C., a correction must be applied to the standards as well as to the dialysate to obtain the true pH at 20°C. The pH of all the blood dialysates in these data were corrected to 20°C.

Henderson (1908) has shown that the ionization constants of carbonic acid and the ion $H_2PO_4^-$ in the equilibria $\frac{NaH_2PO_4}{Na_2HPO_4}$ and $\frac{H_2CO_3}{NaHCO_3}$ increase upon raising the temperature from 18 to 38°C. However, the ionization constant of water increases much more rapidly than that of carbonic acid, which produces a large increase in hydroxyl ion concentration with very little change in the concentration of hydrogen ions. Therefore, solutions containing bicarbonate and carbonic acid at a constant carbonic acid concentration increase in alkalinity with a rise in temperature almost as much as the water constant itself, provided there is no loss of bicarbonate by reaction with other substances in the solution. He concludes that this applies to blood. Disregarding the unknown effect of the change of temperature on the dissociation of the indicator, the data of Table I on the standard phosphate solutions agree in the direction of the change with Henderson's work. Triplicate series of standards under oil as used in the blood

determinations were brought to the indicated temperatures and compared. The results are given in Table I.

Changing the tubes from 37 to 10°C. and *vice versa* gave the same results. Therefore, a temperature correction of pH 0.004 per 1°C. was applied to all readings of the standards at temperatures other than 20°C.

Hasselbalch (1917) published extensive data on the effect of changes of temperature on $NaHCO_3$ solutions and blood. He determined the pH of $NaHCO_3$ solutions at 38 and 18°C. at various CO_2 tensions by the electrometric method. The curves plotted as pH against millimeters of tension of CO_2 show a difference between the two temperatures of pH 0.12 at the same CO_2 tension, or a difference of about 12 mm. of CO_2 tension at the same pH. He ascribed this more alkaline reaction at 38°C. to the difference in solubility of CO_2 (0.928 at 18°C. and 0.560 at 38°C.) being greater than the change in ionization constant of the carbonic acid. Serum gave results similar to the bicarbonate. At the same CO_2 tension it was pH 7.39 at 18°C. and pH 7.49 at 38°C., a difference of pH 0.10. The results on whole blood differed from those of the serum and bicarbonate in that the same reaction, pH 7.35, was found at both temperatures. Hasselbalch surmised a greater acid dissociation of oxyhemoglobin at 38 than at 18° as the explanation of this phenomenon in the whole blood. Another factor, however, is suggested by the following calculations. Plotting the curves at 38 and 18° from Hasselbalch's figures[1] and calculating the pH at 40 mm. tension, the following figures are obtained:

TABLE I.

Temperature Changes of Standard Phosphate Buffers.

Buffer No.	Temperature.		
	10°C.	23°C.	37°C
	pH	*pH*	*pH*
1	7.15	7.20	7.25
2	7.25	7.30	7.35
3	7.35	7.40	7.45
4	7.45	7.50	7.55

[1] Hasselbalch (1917), Blood I, Table II, p. 123.

$$\text{pH at } 38° = 6.09 + \log \frac{40.31}{2.69} = 7.27$$

$$\text{pH at } 18° = 6.19 + \log \frac{49.0}{4.50} = 7.23$$

The equations show that there were 49.0 volumes per cent of combined CO_2 at 18° and 40.31 volumes per cent at 38°or an increase of 8.69 volumes per cent of combined CO_2 with the decrease of 20° in temperature. If the pH at 18° is calculated with the same amount of combined CO_2 as was found at 38° (40.31 volumes per cent), the

$$\text{pH at } 18° = 6.19 + \log \frac{40.31}{4.50} = 7.14$$

or pH 0.13 more acid at 18 than at 38°. This difference of pH 0.13 for whole blood is about the same as that found with bicarbonate and serum between 18 and 38°. These calculations would indicate that the red blood cells made available a larger amount of alkali at 18 than at 38°, which was probably due to the increased amount of dissolved CO_2 causing a shift of Cl^- from the plasma into the cells (Van Slyke, 1921). In this way the increase in available alkali which accompanied the decrease in temperature balanced the increase in CO_2 solubility and the decrease in acid dissociation so that the reactions at the two temperatures were nearly the same.

Other investigators disagree with Hasselbalch. McClendon (1917) reported that if the CO_2 tension remained constant, the pH of bicarbonate solutions, sea water, or blood varied with the temperature, pH 0.01 for each degree Centigrade. Evans (1921) concluded that at the same CO_2 pressure human blood was pH 0.20 more alkaline at 38 than at 20°.

Our data on the temperature factor for correcting the pH of the dialysate of whole blood are not exactly comparable to those quoted above as regards a constant tension of CO_2. Where the change in temperature in a solution under oil containing CO_2 is a cooling one, as in the case of blood from body to room temperature, the solubility of the CO_2 is increased, which should minimize any loss of dissolved CO_2 from the solution. It seems probable that there is no change in amount of dissolved CO_2 with a decrease in temperature from 38 to 20°C., but that the increased solubility of CO_2 at 20° makes the change comparable to a decrease in

tension. This is illustrated by the following results from a pure $NaHCO_3$ solution. A solution of $NaHCO_3$ (0.0267 M) was equilibrated with 46.0 mm. of CO_2 at 37°C. and run into tubes under oil without exposure to the atmosphere. One-half of the solution was kept in the equilibrating room at 37° and the other cooled to 20°. The pH of each was determined by adding the indicator directly to the clear solution. Their colorimetric reaction was pH 7.39 at 37°C. and pH 7.37 at 20°C. The total CO_2 content of the solution was determined in the Van Slyke apparatus and the following data obtained for 37°C.

	vol. per cent
Total CO_2 content	62.2
Dissolved CO_2 $(\frac{46}{760} \times 0.560)$	3.39
$NaHCO_3$	58 81

pH = 7.31 (Hasselbalch's value for pK_1).
pH = 7.39 (Warburg's " " ").

If it is assumed that on cooling no change in amount of dissolved CO_2 is involved, then at 20°C. the solution would contain 3.39 volumes per cent of dissolved CO_2 with a solubility of approximately 0.900 (interpolated from Hasselbalch, 0.928 at 18°). The CO_2 tension at 20°, calculated from the equation

$$\frac{CO_2 \text{ tension}}{760} \times 0.900 = 3.39$$

equals 28.6 mm. or 17.4 mm. lower than the CO_2 tension of the same solution at 37°C. According to Hasselbalch's equation, the reduction in CO_2 tension between pH 7.39 at 38°C. and pH 7.37 at 20° should be approximately 8 mm. instead of 17.4 mm. Whether this discrepancy was due to technical error or was a valid one was not determined. These data would indicate, however, that if a bicarbonate solution with a molar concentration and CO_2 tension of the order of blood is kept under oil, a reduction in temperature from 38 to 20°C. produces a reduction in CO_2 tension which tends to counteract the change in ionization of the carbonic acid and involves but little change in the hydrogen ion concentration.

To determine the temperature correction factor for the pH of the dialysate of whole blood the following experiments were conducted. Duplicate sets of dialyzing tubes were prepared and

brought to the two desired temperatures, incubator and room temperature or room temperature and ice bath. Immediately after drawing the venous blood, equivalent dialyses were made at the two temperatures. The color comparisons with the standards were made at the dialyzing temperatures. The results are given in Table II.

Each pH figure represents the average of triplicate dialyses. In the last determination, Blood 186, the blood was drawn in the 38° incubator room and the 38° sample was not exposed to any change of temperature. The table shows that dialysis at the lower temperatures, 13 to 28°C., always gave a more acid reaction than dialysis at the higher temperatures, 26 to 38°C. The difference was fairly consistent with five different bloods and averaged

TABLE II.

Dialysis of Blood at Different Temperatures.

Blood No	Temperature in degrees Centigrade.										Difference in temperature.	Difference in pH.
	38	32	31	28	27	26	20	19	16	13		
	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH	°C.	
163			7.50				7 38				11	0.12
164		7.43						7.33			13	0.10
181						7 40			7.29		10	0.11
183					7.28					7.10	14	0 18
186	7.16			7.10							10	0.06
Total											58	0.57

approximately pH 0.01 for each degree Centigrade or pH 0.18 more acid at 20°C. than at 38°C.

The explanation of these results and comparison with other data on changes in whole blood according to variations in temperature is complicated by two factors of unknown value: first, a possible change in CO_2 tension when the blood is cooled in the test-tube under oil, and second, the effect on the reaction of the dialysis and the accompanying dilution with physiological salt solution. In regard to the first factor, there is probably little if any loss of total CO_2 from the blood when cooled under oil (Joffe and Poulton, 1920). If the decrease of pH 0.20 (Table II) between 38 and 18° is applied to Hasselbalch's blood which has a total CO_2 at 38° of 43.0 volumes per cent at 40 mm. tension,

the distribution of CO_2 between dissolved and combined CO_2 at 18° can be calculated as follows:

$$\text{pH at } 38° = 7.27 = 6.09 + \log \frac{43.00 - 2.69}{\frac{40}{760} \times 0.511}$$

$$\text{pH at } 18° = 7.07 = 6.19 + \log \frac{43.00 - 5.00}{\frac{44.5}{760} \times 0.854}$$

The equations show that in changing from pH 7.27 at 38° to pH 7.07 at 18° without changing the total CO_2, there would be a decrease in $NaHCO_3$ from 40.31 to 38.0 volumes per cent and an increase in dissolved CO_2 from 2.69 to 5.00 volumes per cent with an increase in CO_2 tension from 40 to 44.5 mm. Such a change might represent the final equilibrium attained after a shift of HCl between cells and plasma, initiated by a tendency to decrease in CO_2 tension with decrease in temperature as illustrated in the pure $NaHCO_3$ solution. It seems more probable that the second factor, dialysis, plays some part in the change. A discussion of Donnan's membrane equilibrium theory as applied to the dialysis of whole blood is given in the succeeding article.[2] The difference between the pH of the plasma and that of the dialysate is explained by the theory as due to the effect of the non-diffusible ions on the distribution of the diffusible ions between the three phases, red corpuscle, plasma, and dialysate. Inasmuch as no experimental data have been found on the influence of temperature changes on the equilibrium, it is deemed sufficient to note here that in the change from 37 to 20° involving a change in the dissociation constants of the salts and colloids of the blood it is quite probable that the equilibria between diffusible and non-diffusible ions are altered.

A third factor, the loss of CO_2 by diffusion through the oil, was determined on the 0.0267 M bicarbonate solution as follows. One sample of the equilibrated $NaHCO_3$ was run by replacement with oil directly from the equilibrator through a small bore tube into a comparator tube containing the indicator, and the pH read immediately. This sample, unexposed to oil, gave a reaction at 37° of pH 7.39. Duplicate sets of tubes containing the $NaHCO_3$

[2] Chambers, W. H., and Kleinschmidt, R. E., *J. Biol. Chem.*, 1923, lv, 257.

under oil, without exposure to the air, were kept at 37 and 20°C. for 1 hour and the pH was read at intervals as indicated.

Time after equilibration.	37°C.	20°C.
	pH	*pH*
Immediately	7.39	7.37
15 minutes	7.42	7.37
30 "	7.45	7.39
60 "	7.47	7.39

From these figures it is apparent that an appreciable loss of CO_2 from the solution takes place at 37°C. but no loss was detected at 20°C. for 15 minutes, which was the time and approximate temperature used in the blood dialyses.

The data of Table II do not substantiate the statement of Evans (1921) that if the blood is equilibrated at 38° the dialysis gives the same results as at room temperature. Evans cites Joffe and Poulton (1920) who state that cooling the blood under oil before centrifuging caused no definite change in the total CO_2 of the plasma. However, no change in the total CO_2 of the plasma does not necessarily mean no change in pH, for the pH of the blood is not a function of the distribution of total CO_2 between cells and plasma but of the ratio of combined CO_2 to dissolved CO_2.

The temperature change for whole blood of pH 0.01 per degree Centigrade determined from the data of Table II is used as a correction factor in the calculation of all samples of whole blood not dialyzed at 20°C. The factor of pH 0.004 per degree Centigrade was applied to the standards when they were read at a temperature other than 20°C. The salt error of the cresol red indicator which was applied to all dialyses was determined for 0.8 per cent NaCl as + pH 0.06. Therefore, the corrections to 20°C. for the dialysate of whole blood dialyzed and read at 25°C. as pH 7.35 would be 7.35 + 0.06 (salt error) −0.02 (correction for K_2HPO_4 standards) −0.05 (temperature correction for whole blood dialysis) = pH 7.34 at 20°C.

EXPERIMENTAL DATA.

The main experimental work consists of the hydrogen ion concentration determination of the dialysate from 92 specimens of venous blood. The blood was obtained from patients with car-

cinoma, from those with other diseases, such as non-malignant growths, anemia, tuberculosis, and lues, and from normal subjects.

The normal control bloods were given for the most part by students of the Medical School. Table III shows the results obtained on twenty-three specimens from fourteen normal subjects. The blood samples were numbered consecutively when drawn, so that the first column (blood number) in each table indicates the order in which the bloods were taken. As the numbers of Column 1 show, the normal controls were selected at intervals during the entire period of investigation of the other

TABLE III.

Hydrogen Ion Concentration of the Dialysate of the Blood of Normal Subjects.

Blood No.	Subject.	pH at 20°C.	Blood No.	Subject.	pH at 20°C.
53	J.	7.30	127	K.	7.45
54	W.	7.25	137	"	7.21
55	S.	7.32	144	"	7.38
56	H.	7.35	152	"	7.33
143	B.	7.39	82	C.	7.30
157	D.	7.30	125	"	7.43
174	M.	7.36	138	"	7.10
175	A.	7.37	139	"	7.28
177	R.	7.18	153	"	7.34
178	V.	7.20	164	"	7.34
179	N.	7.30	167	"	7 32
182	R.	7.35			
Average..					7.31

cases, so that any slight improvement in technique or variation in other factors during the course of the work is checked in this way. The average of the twenty-three determinations listed in Table III is pH 7.31, with a variation of pH 7.10 to 7.45. The variation in a single individual is shown by the four different specimens from Subject K and seven from Subject C. Five of the seven tests of Subject C show a pH of 7.31 ± 0.03. The other two, Nos. 125 and 138, might be considered as temporary or local deviations from the normal zone, magnified by experimental error.

For convenience in presentation, the carcinoma cases are divided into five tables according to the location of the initial lesion. The following items concerning each case are tabulated: blood num-

TABLE IV

Hydrogen Ion Concentration of the Dialysate of the Blood in Carcinoma of the Head Region.

Blood No.	Clinical diagnosis	Pathological diagnosis	Extent or duration of disease	Treatment prior to test	Course	pH at 20°C
181	Carcinoma of left ear.	Prickle cell carcinoma.	Anterior auditory canal, no glands involved.	None.	Prognosis good.	7 38
87	Carcinoma of tongue.	Malignant squamous cell carcinoma.	Lesion 1×1.5 cm. Lymph glands not involved (hyperplasia).	Wassermann ++++. Antiluetic, intensive for 3 weeks.		7 40
172	Carcinoma of lower lip.	Prickle cell carcinoma, lymph glands negative.	Ulcer 3 cm. in diameter.	None.	Prognosis good.	7 47
106	Carcinoma of right maxilla.	Malignant squamous cell carcinoma.	Lesion 2×4 cm.	None.	Erysipelas 2 weeks later.	7 45
88	Carcinoma of left ear.	Malignant squamous cell carcinoma.	Lesion 4×7 cm. of 10 years duration.	None.	Improved.	7 50
96	Carcinoma of left antrum.	Malignant squamous cell carcinoma.	Entire left upper jaw involved, extensive scarring.	Wassermann ++++. Antiluetic, 2 months.		7 30
77	Carcinoma of left antrum.	Malignant squamous cell carcinoma.	Left maxilla and left cervical glands. Glands 8×5×3 cm.	Glands only, removed 11 days prior.	Died 3 months later.	7 51

105	Carcinoma of lower lip.	Malignant squamous cell carcinoma with prickles.	Lip 3×6 cm. Glands 5×8×4 cm. and 3×4 cm.	None.	Died of broncho-pneumonia 2 months later.	7.40
70	Carcinoma of left tonsil.	Malignant squamous cell carcinoma.	Involving regional lymph glands.	None.	Died 14 days later.	7.35
Average				...	. .. .	7.42

TABLE V.

Hydrogen Ion Concentration of the Dialysate of the Blood in Carcinoma of the Breast.

Blood No.	Clinical diagnosis.	Pathological diagnosis.	Extent or duration of disease.	Treatment prior to test.	Course.	pH at 20°C.
180	Carcinoma of right breast.	Medullary carcinoma, glands negative.	10 months duration. Breast enlarged twice.	None.	Favorable.	7.26
151 154	Carcinoma of left breast.	Medullary carcinoma, metastases to axillary glands.	Breast tumor 12×8×6 cm. Gland 4×4×4 cm. 5 years duration.	None.		7.32 7.42
165	Carcinoma of left breast.	Adenocarcinoma, metastases to lymph glands.	Breast tumor about 2 cm. in diameter. Axillary gland 3×3 ×2 cm. 3 months duration.	None.	Favorable.	7.47
128	Carcinoma of right breast.	Adenocarcinoma.	Metastases to axilla.	Right breast entirely gone from plasters.		7.45
58	Carcinoma of right breast.		Axillary and mediastinal lymph glands. Elephantiasis of right arm.		Died 2 weeks later.	7.48
115	Carcinoma of left breast.		Tumor 5×3 cm. extending into axilla. Metastases to vertebræ and pelvis.	Radical right breast operation 7 months previous.		7.44

80	Carcinoma of right breast.	Medullary carcinoma.	Metastases to axilla and under clavicle.	None.	4 weeks later many nodules in axilla and on arms.	7.49
84				7 days after radical breast operation.		7.50
110				1 month after radical breast operation.		7.45
133				2 months after radical breast operation. 1 week after x-ray.		7.51
59	Bilateral carcinoma breast.		Skeleton, lungs, and pleura involved.	1 x-ray treatment.	Died 2 months later.	7.55
113				5 " treatments to thighs.		7.47
Average						7.45

TABLE VI.

Hydrogen Ion Concentration of the Dialysate of the Blood in Carcinoma of the Thoracic and Abdominal Organs.

Blood No.	Clinical diagnosis.	Pathological diagnosis.	Extent or duration of disease.	Treatment prior to test.	Course.	pH at 20°C.
150	Carcinoma of sigmoid.	Adenocarcinoma of sigmoid.	Papilloma 1 cm. in diameter. Musculature not involved.		Favorable.	7.32
119	Carcinoma of rectum.	Adenocarcinoma of rectum.	Ulcerous mass of tumor tissue 1 cm. thick.	Colostomy 14 days and radium 6 days prior.	Favorable.	7.37
65	Carcinoma of intestine.	Annular adenocarcinoma of intestine.	Small mass in sigmoid. Glands not involved.	Tested during saline hypodermoclysis.		7.33
112	Carcinoma of pancreas.		No lymphatic enlargement. Jaundice.		Died 2 weeks later, cardiac failure.	7.51
92	Recurrent carcinoma of left arm.	Squamous cell carcinoma with metastases to lymph glands.	Ulcer deltoid region 4×8 cm.		Questionable.	7.45
142	Recurrent carcinoma of rectum.	Adenocarcinoma of rectum, chronic inflammation at anus.	Mass filling pelvis, inguinal and femoral glands.	Colostomy and excision of rectum 1 year prior.	Died 3 months later.	7.34
66	Carcinoma of intestine.	Adenocarcinoma.	Occupies entire hepatic flexure, part of ascending colon, glands, and right kidney. Not in liver.		Died 18 days later.	7.38

132	Carcinoma of pancreas and liver.		Head of pancreas, wall of duodenum. Liver studded with nodules. Jaundice.	None.	Died 3 months later.	7.41
155				Cholecystectomy 1 month prior.		7.35
85	Carcinoma of pancreas and liver.	Adenocarcinoma.	Pancreas, liver, retroperitoneal glands. 3 months duration.			7.65
72	Carcinoma of ascending colon.	Colloid carcinoma.	Ascending colon, large mass in omentum, liver studded. 6 months duration.			7.60
131	Carcinoma of stomach.		Entire stomach involved.		Died 19 days later.	7.53
122	Carcinoma of lung.	Carcinoma of right lung, pleura, chest wall, and diaphragm.	Right lung primary, metastases to left pleura, liver, adrenals, and ovary.		Died 2 months later.	7.45
Average						7.44

TABLE VII.

Hydrogen Ion Concentration of the Dialysate of the Blood in Carcinoma of the Pelvic Organs.

Blood No.	Clinical diagnosis.	Pathological diagnosis.	Extent or duration of disease.	Treatment prior to test.	Course.	pH at 20°C.
71	Carcinoma of cervix uteri.	Gelatinous degeneration of carcinoma of the uterus.	Limited to cervix.	Radium 6 days prior.		7.40
173	Carcinoma of uterus.	Carcinoma.	Parametria, anterior and posterior fornix involved.	Radium and x-ray 3 months prior.	Favorable.	7.43
121	Carcinoma of peritoneum, ovarian cyst.	Malignant papillary cyst,—adenoma of ovary.	Most of peritoneum. Cystic ovary and pelvic organs removed 6 years previous.	X-ray during 2 years prior.		7.55
162				2 gallons fluid drained from peritoneum 3 days prior.		7.36
130	Carcinoma of prostate.	Adenocarcinoma of prostate gland.	Complete prostate involved.	Radium and cystostomy 7 days prior.	Prostatectomy. Died 2 days later.	7.53
126	Carcinoma of clitoris.	Prickle cell carcinoma of clitoris with glandular metastases.	Initial tumor 1×2 cm. Inguinal and femoral glands involved later.	None.	Died 8 months later.	7.58
Average......						7.47

TABLE VIII.

Hydrogen Ion Concentration of the Dialysate of the Blood in Inoperable Carcinoma of the Cervix.

Blood No.	Clinical diagnosis.	Pathological diagnosis.	Extent or duration of disease.	Treatment prior to test.	Course.	pH at 20°C.
63	Inoperable carcinoma of uterus.		Recurrent 9 months after Percy operation.	Radium 1 day prior.		7.63
134	Inoperable carcinoma of cervix uteri.	Adenocarcinoma, glandular hyperplasia, chronic endometritis.	Very extensive.		Favorable with radium.	7.45
146	Inoperable carcinoma of cervix uteri.		Very extensive.		Questionable.	7.46
124	Inoperable carcinoma of cervix uteri.		Metastases to parametria very extensive.	Radium 6 days prior, x-ray 4 days prior.	Died 5 months later.	7.53
Average						7.52

TABLE IX.

Hydrogen Ion Concentration of the Dialysate of the Blood in Diseases Other than Carcinoma.

Blood No.	Clinical diagnosis.	Pathological diagnosis	Description.	pH at 20°C.
114	Pernicious anemia.		R.B.C. (after transfusion) 2,740,000. Hb. 65 per cent.	7.37
73	Simple anemia, myelogenous leucemia.		R.B.C. 3,520,000. Hb. 65 per cent. W.B.C. 165,400.	7.35
135	Myelogenous leucemia.		R.B.C. 2,890,000. Hb. 65 per cent.	7.35
64	Traumatic hematoma.			7.34
60	Fibroid uterus.	Fibroid uterus multiple.		7.33
161	" "	Chronic inflammation and granulation tissue.		7.35
160	" "	Intramural fibroids.		7.33
109	Verruca on left buttock.	Fibroma with chronic inflammation.		7.40
74	Hyperplasia of epithelium.		Lower lip.	7.40
163	Carcinoma of cervix.	Acute and chronic cervicitis.		7.40
183	Epithelioma.	Chronic inflammation, epithelial hyperplasia.		7.24
129	Chronic pulmonary tuberculosis.	Tubercle bacilli in sputum.		7.40
145	Tuberculosis of rectum and lungs.*	Tuberculosis.		7.43
156				7.41
117	Ulcer of duodenum.			7.42
140	Lues.		Wassermann ++++.	7.14
141				7.27

107	Lues.		Wassermann ++++.	7.32
83				7.40
98	Exfoliative dermatitis.			7.37
104				7.35
111	Exfoliative dermatitis.			7.35
108				7.48
81	Adhesions of peritoneum.		Postoperative.	7.50
Average				7.36

* Numbers enclosed in a bracket are separate determinations on different days on the same patient.

ber, clinical diagnosis, pathological diagnosis, comparative size of the cancer or its duration as an indication of its malignancy, remarks concerning any treatment or condition of the patient which might influence the reaction of the blood, the course of the disease if known, and the pH of the dialysate of the whole blood at 20°C. Clinical data were taken from the hospital records of the patients.

Nine cases of carcinoma of the head region are reported in Table IV. The variation in the pH of the dialysate is quite wide, pH 7.30 to 7.51. The average is pH 7.42, which is pH 0.11 more alkaline than the normal average of Table III. Table V gives the results from thirteen determinations on eight cases of carcinoma of the breast. The range of reaction is greater in this table, pH 7.26 to 7.55, and the average more alkaline, pH 7.45 or pH 0.14 higher than the normal. Table VI shows thirteen determinations on eleven cases of carcinoma of the thoracic and abdominal organs including the intestinal tract, and one carcinoma of the arm. The variation is from pH 7.32 to 7.65, with an average of pH 7.44. Tables VII and VIII contain the cases of carcinoma of the pelvic organs, those in Table VIII being inoperable carcinoma of the cervix and uterus. The average of the six cases of Table VII is pH 7.47. However, it should be noted that Blood 162 was drawn 3 days after 2 gallons of fluid had been drained from the cysts in the peritoneal cavity. As the effect on the blood of this loss is unknown, this determination might be excluded, giving an average for the five cases of pH 7.50, and a range of pH 7.40 to 7.58. The average of the four cases in Table VIII is pH 7.52.

The miscellaneous pathological cases, or diseases other than carcinoma are grouped in Table IX. These include three cases of anemia, several of non-malignant growths and inflammation, two cases of tuberculosis, and several cases from the Dermatological Service. The average of the twenty-four cases reported in Table IX is pH 7.36, only pH 0.05 more alkaline than the normal average. The variation is similar to the normal, being pH 7.14 to 7.50.

The averages for the different groups are assembled in Table X.

Table X shows that the averages for the carcinoma cases are distinctly more alkaline than those of the normal and miscellaneous cases.

The comparison of the carcinoma cases with the cases having other diseases (miscellaneous pathological) and with the normal subjects is graphically shown in Fig. 2. Each point represents a

TABLE X.

Average Hydrogen Ion Concentration of the Dialysate of the Blood.

Table No.	Disease.	No. of determinations.	Average pH at 20°C.
III	Normal.	23	7.31
IX	Miscellaneous pathological.	24	7.36
IV	Carcinoma of head.	9	7.42
VI	" " abdominal organs.	13	7.44
V	" " breast.	13	7.45
VII	" " pelvic organs.	6	7.47
VIII	Inoperable carcinoma of cervix.	4	7.52

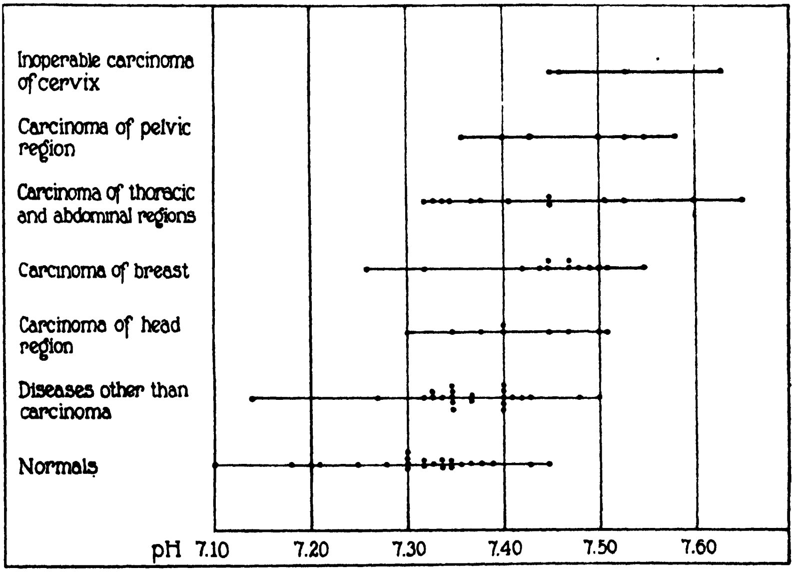

FIG. 2. The hydrogen ion concentration of the dialysate of the venous blood.

pH determination, given in the data of Tables III to IX. The expression of the pH is a linear one but the cases are grouped along several lines, divided according to tables, to avoid the confusion of crowding the points onto one line. In this way

Fig. 2 shows at a glance that the majority (two-thirds) of the normal and miscellaneous cases lie in the zone between pH 7.30 and 7.40, whereas the majority (two-thirds) of the carcinoma cases have a pH above 7.40. It is evident then, that with some exceptions the dialysate of the whole blood of the patients with cancer is more alkaline than that of normal persons or patients with the other diseases studied.

DISCUSSION.

Menten (1917) found no discernible relationship between the alkalinity of the blood serum and the degree of anemia in cancer patients. Barr and Peters (1921) report six cases of anemia in which the arterial blood was markedly alkaline, but the venous blood had a normal reaction varying between pH 7.32 and 7.38. Similar results are found in the dialysate from the venous blood of the three cases of anemia reported in Table IX (Nos. 114, 73, and 135) which gave respectively, pH 7.37, 7.35, and 7.35. Many of the carcinoma cases were anemic, however, no correlation was found between the red corpuscle count or hemoglobin determination and the reaction of the dialysate. It seems from this evidence that the anemia of cancer patients is not a factor in producing the more alkaline reaction of the blood dialysate.

The cases of non-malignant growths given in Table IX show no definite change in pH from the normal zone. The three determinations on two cases of tuberculosis were pH 7.40, 7.43, and 7.41. The data indicate that the more alkaline reaction found in the majority of the cases with malignant growth is associated definitely with the carcinoma.

Some indication of the nature of the relationship between the tumor and the hydrogen ion concentration of the blood dialysate is found in a study of the data of Tables IV to VIII. The cases have been arranged in the tables in the order of increasing size or malignancy of the tumor. For example, in Table V, in the first case (No. 180) the carcinoma was confined to the breast and the glands were not involved. In the next three cases (Bloods 151, 154, 165, and 128) there were metastases to the axillary glands, and in the cases following there were more extensive metastases to other parts of the body. The last case (Bloods 59 and 113) was carcinoma of both breasts with metastases through-

out the body. This arrangement of cases applies to all of the carcinoma tables. Its accuracy is, of course, only approximate, for the actual area of the growing tumor can be only estimated, but it is based on a careful compilation of the clinical and pathological examinations. Reviewing all of the data, there seems to be an increase in alkalinity with an increase in the size and extent of the tumor growth. This is particularly well illustrated in Table V. The first case in Table V shows a pH of 7.26, with the intermediate cases between pH 7.42 and 7.50 and the last case pH 7.55. In Table VI, considering only the cases of carcinoma of the intestinal tract, the small tumors show no increase in alkalinity above the normal zone; the cases with metastases, a distinct alkalinity. Blood 66 appears to be an exception, for the extent of the metastases points toward a higher pH than was found. In general the same increase in alkalinity with increased involvement of the different glands or organs is found in the cases of Tables VII and VIII. Table IV, the cases of carcinoma of the head region, presents the greatest number of exceptions. Blood 172 with only a small carcinoma has a pH of 7.47 while Nos. 96, 105, and 70 have quite extensive involvement with reactions of pH 7.30, 7.40, and 7.35, respectively. No explanation of these disagreements has been found. However, Peters, Barr, and Rule (1921) have called attention to the wide variation in venous blood which they found in three normals, pH 7.22, 7.30, and 7.40, and point out that a normal figure for an individual should be established before a change toward the acid or alkaline can be defined, unless it falls distinctly outside the normal zone. Thus a pH of 7.35 or 7.40 might be an alkalosis for certain individuals who have a normal pH of 7.20 or 7.25.

Data on the effect of operative removal of the cancer are meager, for cases in which all of the carcinoma was removed were most often the early cases without metastases which showed no marked alkalinity. One case of carcinoma of the breast gave a reaction of pH 7.37 before operation and 6 days postoperative gave a pH of 7.27, another case of carcinoma of the lip had a pH of 7.47 before operation and a pH of 7.18, 30 days after complete excision of the tumor. Other cases which showed no reduction after operative treatment were later found to have metastases.

These results indicate that with the increasing size of the cancer there is a progressive change in the reaction of the blood dialysate. This may have a very definite clinical value in the prognosis of operative treatment of advanced cases of carcinoma.

SUMMARY.

The hydrogen ion concentration of the dialysate from the venous blood of forty-five cases of carcinoma averaged pH 7.45 at 20°C. This is distinctly more alkaline than that of the normal subjects which averaged pH 7.31.

The pathological cases other than carcinoma gave slightly more alkaline results than the normals, averaging pH 7.36 at 20°C.

No association of anemia with this increase in alkalinity in the venous blood dialysate in cancer was established.

In general the degree of alkalinity corresponded to the size and extent of the tumor growth. Small tumors without metastases showed little or no increase above the normal zone.

Some modifications to prevent the loss of CO_2 were made in the method of dialyzing blood for the colorimetric determination of the hydrogen ion concentration.

Data are given on the changes in pH and CO_2 tension in cooling a bicarbonate solution under oil and on the loss of CO_2 through the oil at different temperatures.

The factors for correcting the temperature to 20°C. were found to be pH 0.004 per degree Centigrade for the phosphate standards and pH 0.01 per degree Centigrade for the blood.

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THE HYDROGEN ION CONCENTRATION OF THE BLOOD IN CARCINOMA.

II. FROM THE CO_2-BICARBONATE RATIO.

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In the first paper of this series,[1] the venous blood of carcinoma cases was compared with normal blood and blood from other diseases as to hydrogen ion concentration of its dialysate. An increase in alkalinity was found in the extensively involved carcinoma cases. If this difference in the dialysate represents an actual change in the hydrogen ion concentration of the blood, certain compensating changes in respiration and excretion might be expected. According to Van Slyke's résumé on normal and abnormal blood reactions (Van Slyke, 1921, *a*) an increase in alkalinity in the blood may be associated with either an uncompensated alkali excess or an uncompensated CO_2 deficit. The former condition has been reported by several observers following overdosing with $NaHCO_3$. The compensation was by retention of H_2CO_3 through a decrease in respiration and by excretion of alkali through the urine. The latter condition, CO_2 deficit, has been experimentally caused by hyperpnea (Grant and Goldman, 1920) as well as noted during a decreased oxygen supply. An uncompensated alkali excess was also produced by obstructing the pylorus and removing the secreted gastric HCl (MacCallum, Lintz, Vermilye, Leggett, and Boas, 1920). This is of interest in connection with the decrease in the total acidity, and especially the HCl, of the gastric juice in cancer, which was observed by Moore, Alexander, Kelly, and Roaf (1906), and confirmed by Palmer (1906). To look for such abnormal variations in base content or CO_2 tension of the venous bloods and to determine

[1] Chambers, W. H., *J. Biol. Chem.*, 1923, lv, 229.

the hydrogen ion concentration of the venous bloods from the CO_2-bicarbonate ratios, for comparison with the colorimetric determinations on the dialysate, a study of the carbon dioxide absorption curves of cancer and normal bloods was made.

Method.

All the blood gas determinations were made on the venous blood sample, drawn and kept under oil as described in the previous paper.[1] The gas mixtures of CO_2, nitrogen, and air for equilibrating the blood were measured into rubber bladders. Approximately 900 cc. of the gas were forced through a 300 cc. separatory funnel, from the small end, leaving the balance from 1,000 cc. in the bag for the CO_2 and O_2 determinations which were made in the Haldane gas analysis apparatus. Analyses comparing the gas remaining in the bag with the gas in the separatory funnel showed a difference of less than 1 mm. of tension of oxygen and CO_2, so that the more rapid and convenient method of analyzing the gas remaining in the bag was adopted.

2 to 3 cc. of blood were pipetted into the funnel immediately before or during the time the gas mixture was forced through it in order to minimize gas changes in the blood. All equilibrations were carried on in a water bath at 37–38°C. for 15 minutes with a mechanical rotator. The gas pressure in the funnel was released to atmospheric pressure at the temperature of the water bath. The blood was measured directly from the separatory funnel at 37° into the Van Slyke apparatus, and the CO_2 or O_2 determined according to the earlier technique (Van Slyke, 1917). The calculations of the results for CO_2 and O_2 are made from Van Slyke's more recent tables (Van Slyke and Stadie, 1921). The CO_2 absorption curves have been plotted according to the usual method with the total CO_2 content of the blood in volumes per cent as ordinates and the CO_2 tension in millimeters of mercury as abscissæ.

The venous CO_2 tensions were determined from the CO_2 absorption curves in two different ways: (1) In the greater part of the experimental work the venous oxygen tension was determined, the CO_2 absorption curve was then constructed at the venous oxygen tension and the venous CO_2 tension determined by

placing the venous point directly on the curve. (2) For the other blood samples the CO_2 absorption curve was made from fully oxygenated blood, so the venous point was located above the curve by allowing for the venous oxygen unsaturation.

Venous Oxygen Tension.

The effect of variations in the CO_2 tension on the O_2 saturation of hemoglobin at different O_2 tensions has been pointed out by Barcroft (1914) and others. As the CO_2 tension of each of the venous blood samples was different, and was unknown at the beginning of the analyses, it was necessary to select an arbitrary tension of CO_2 at which to determine the venous O_2 tension. 50 mm. were taken as a mean CO_2 tension and the O_2 tensions determined accordingly.

At first the oxygen absorption curve for each normal and pathological blood was constructed by plotting the total O_2 content in volumes per cent against the O_2 tension in millimeters. The venous tension was thus read directly from its determined O_2 content. As this method required three to five oxygen determinations the more rapid procedure of determining the venous tension from the percentage saturation of the hemoglobin was adopted. Barcroft (1914) has given the curves for the percentage saturation of hemoglobin with oxygen plotted against the oxygen tension at 40 and 90 mm. of CO_2, but as 50 mm. were the CO_2 tension selected for this work a curve similar to the one in Chart 2 was constructed from a series of determinations on normal blood (Blood 144, Table I), which ranged between 45 and 50 mm. of CO_2. Only two analyses, the O_2 content of the venous blood and that of the fully oxygenated blood, were thus required to calculate the percentage saturation of the venous blood and to read from this on the curve (Chart 2) the O_2 tension of the venous blood at 50 mm. of CO_2. During the course of the experimental work data were obtained on both normal and pathological bloods for other points on the oxygen absorption curve. All of these data have been collected in Table I, and the curve for 50 mm. of CO_2 which is given in Chart 2 was thus made from twenty oxygen analyses.

As the points were determined at different CO_2 tensions (last column of Table I), they are not comparable for the direct drawing

TABLE I.

Oxygen Absorption of Hemoglobin at Different O_2 and CO_2 Tensions.

Blood No.	Diagnosis.	O_2 capacity of blood.	Chemically combined oxygen	Oxygen tension	Percentage saturation.	Value of K by Hill's equation.	CO_2 tension.
		vol per cent	*vol. per cent*	*mm.*			*mm.*
144	Normal.	19.87	5 50	16 0	27.7	0.000374	47.9
144	"	19.87	7.30	17.9	36.7	0.000428	47.7
144	"	19 87	11 95	30.7	60.1	0 000289	45.4
144	"	19.87	15.81	43 3	79.6	0 000316	48.5
144	"	19 87	17 93	76 2	90.2	0 000312	48.2
152	"	20.77	13.89	28.3	66.9	0.000474	47.9
152	"	20 77	17.66	47.7	85.0	0.000361	47.9
157	"	18 40	15.30	40.7	83.2	0.000469	47.5
177	"	20 55	7.64	20 9	37.2	0 000255	60.3
182	"	22 33	7.47	17.2	33.5	0.000410	37.2
182	"	22.33	11.26	25.0	50.4	0.000325	61.4
153	"	20 53	14 54	35.8	70.8	0.000316	45.8
145	Tuberculosis.	10.87	3 39	18.8	31.2	0.000296	46.4
161	Fibroid uterus.	19.87	7.49	19.6	37.7	0 000355	46.5
147	Carcinoma bladder.	15 97	6 19	19.3	38.4	0 000385	46 4
147	" "	15.97	11.85	34.2	74.2	0.000421	47.7
165	" breast.	17.47	9 67	24 0	55.4	0.000440	47.5
151	" "	13.03	4.89	20 5	37.5	0 000316	47.0
148	" bladder.	10.77	4.10	18.7	39.0	0.000422	46.0
158	Sarcoma uterus.	6.57	3 17	25.6	48.3	0.000282	46.4

of the curve at 50 mm. of CO_2. This curve was accurately constructed from these data, however, by determining the value of K in Hill's equation,

$$\frac{Y}{100} = \frac{Kx^n}{1 + Kx^n} \quad \text{where}$$

Y = percentage of saturation of hemoglobin with O_2,
x = mm. tension of O_2,
K = equilibrium constant, and
n = aggregation of hemoglobin molecules.

These values of K as computed for each point are given in Column 7 of Table I and the values for $\frac{1}{K}$ plotted against millimeters of CO_2 in Chart 1.

From Curve 1 of Chart 1 the value for $\frac{1}{K}$ at 50 mm. of CO_2 is 2,860 and of K is 0.00035. Using this figure (K = 0.00035) the

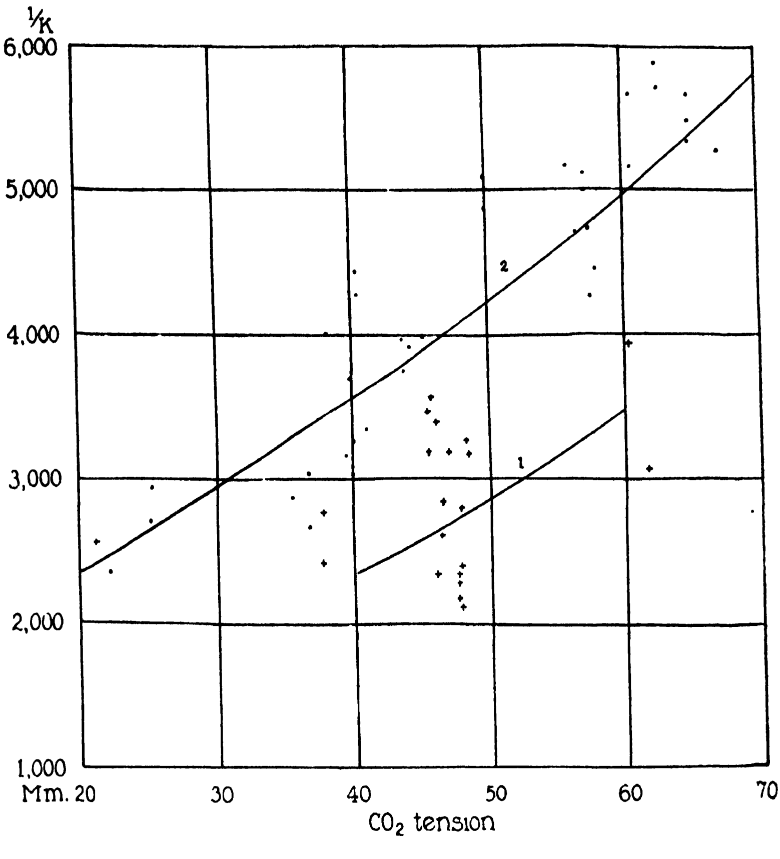

CHART 1. The variation of $\frac{1}{K}$ with the CO_2 tension.

Curve 1 from points designated by a plus (+) sign, data of Table I; Curve 2 from points designated by a dot (·), data of Barcroft et al. (1922).

The recently published extensive data of Barcroft and his associates (1922) have appeared since the experimental work in this paper was done. The value of K (Table I) was calculated on the basis of n = 2.5, whereas 2.2 seems preferable. However, substituting 2.2 for 2.5 in the formula changes the absolute value of K but not the position of the curve in Chart 2. The discrepancy between their value for K at 50 mm. of CO_2 and ours does not seriously affect the results in this paper as it only changes the venous pH value approximately pH 0.01.

curve in Chart 2 was drawn and the points placed on it to show their proximity.

Having determined the venous O_2 tension, the blood was equilibrated with different gas mixtures of CO_2, nitrogen, and air, and the CO_2 absorption curve was constructed at the venous O_2 tension. The venous CO_2 tension could then be read directly

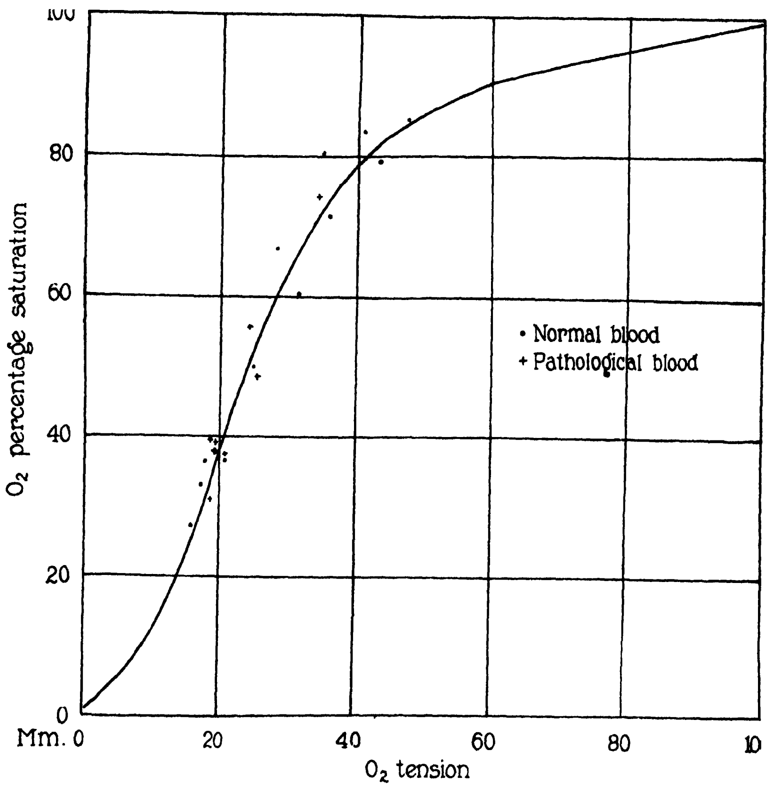

CHART 2. The oxygen saturation of the blood at 50 mm. tension of CO_2.

from the CO_2 absorption curve. Two workers, one to mix and analyze the equilibrating gases, and the other to run the equilibrator and determine the O_2 or CO_2 in the blood while a second sample was equilibrating, after some practise were often able to complete the analyses within 2 hours after the blood was drawn. The order of analyses is best illustrated from one of the protocols; Blood 165.

	Order of procedure.	
1	Determination of chemically combined O_2 in venous blood, *vol. per cent*	9.87
2	Determination of total CO_2 content in venous blood, *vol. per cent*	50 8
3	Equilibration with 132 mm. O_2 and 48.4 mm. CO_2 and determination of chemically combined O_2; *vol. per cent*	17.47
4	Calculation of percentage saturation of venous blood, *per cent*	56.5
5	O_2 tension of venous blood from Chart 2, *mm*	26.5
6	Equilibration with 47.6 mm. CO_2 and 25.8 mm. O_2 and determination of total CO_2 content, *vol. per cent*	51.7
7	Equilibration with 40.6 mm. CO_2 and 23.0 mm. O_2 and determination of total CO_2 content, *vol. per cent*	49.0
8	Venous CO_2 tension from CO_2 absorption curve (Curve 6 and 7), *mm*	45.0

This protocol of Blood 165 also illustrates the theoretical inexactitude of this method, in that the O_2 tension of the venous blood is determined at 50 mm. of CO_2, whereas subsequently it is determined that the CO_2 tension of the venous blood is 45 mm. However, a difference of 5 mm. of CO_2 reduces the O_2 tension of the venous blood approximately 0.4 mm. (Chart 2). A reduction of 0.4 mm. of O_2 is equivalent to raising the CO_2 absorption curve approximately 0.04 volume per cent, which is too small an amount to detect by the methods used. The error, therefore, involved in this technical discrepancy is of no practical significance.

Oxygen Unsaturation.

The earlier part of the work was done before Peters, Barr, and Rule (1921) had called attention to the error of placing a venous point of blood unsaturated with oxygen on a CO_2 absorption curve of fully oxygenated blood. The CO_2 absorption curves for the data of Table VII were also made from completely oxygenated blood, so that for both these groups of bloods (Nos. 113 to 134, and Nos. 167 to 182) the venous point was located above the curve by calculating the oxygen unsaturation of the venous blood in terms of volumes per cent of CO_2 according to the ratio $\frac{CO_2}{O_2} = 0.27$.

The figures for the determination of the value of 0.27 for the ordinate correction and the details of its application in locating the venous point are given in another publication (Doisy, Briggs, Eaton, and Chambers, 1922).

As no oxygen analyses were made on the venous bloods of samples numbered between 113 and 134, the venous points can be placed only approximately. On the twenty-four cases in Tables II to VII (excluding Table III) on which the oxygen capacity of the blood and the oxygen content of the venous blood were determined, the average oxygen unsaturation of the venous blood was 6.72 cc. of oxygen. $6.72 \times 0.27 = 1.8$ volumes per cent of CO_2, which is the average ordinate correction for these twenty-four cases. As the conditions imposed upon the patients before drawing the blood; such as preliminary period of rest, length of time after eating, etc., were the same for all the cases given in Tables II to VII (excluding Table III), this average correction was applied to the blood samples upon which no oxygen determinations (Nos. 113 to 134) had been made, and the venous points were placed 1.8 volumes per cent of CO_2 above the fully oxygenated curves. While the use of an average figure for oxygen unsaturation is entirely unsatisfactory from the standpoint of accuracy, it probably introduced an error not greater than pH 0.03. The results on these bloods are included in the tables and averages, as they give an indication of the order of the venous blood reactions for comparison with those on which the oxygen determinations were made.

Having determined the total CO_2 content and the CO_2 tension of the venous bloods the pH was calculated from the well known formula of Hasselbalch (1917).

$$pH = pK_1 + \log \frac{NaHCO_3}{H_2CO_3}$$

with the following values,

$pK_1 = 6.15$ (Van Slyke, 1922)

$NaHCO_3 = \text{total } CO_2 - H_2CO_3$

$H_2CO_3 = \text{mm. } CO_2 \times 0.0672$ (Van Slyke, 1921, *b*)

EXPERIMENTAL DATA.

Normal Subjects.

The experimental data are presented in the form of six tables (Tables II to VII, inclusive) and seven charts (Charts 3 to 9,

inclusive). In the tables are given the blood number, the diagnosis of the patient if other than normal, the complete oxygen data and venous CO_2 content for locating the venous point on the CO_2 absorption curve, the venous CO_2 tension determined therefrom, and the pH of the venous blood calculated from its CO_2 content and CO_2 tension. The CO_2 absorption curves are given on the charts and the location of each venous point in respect to the curve is also shown. The various oxygen tensions at which

TABLE II

Oxygen, Carbon Dioxide, and pH of the Venous Blood at 38°C. of Normal Subjects.

Blood No.	Subject	O_2 capacity of blood	Chemically combined O_2 of venous blood	Percentage saturation of venous blood	Venous O_2 tension	Venous CO_2 content	Venous CO_2 tension	pH
		vol per cent	*vol per cent*		*mm*	*vol per cent*	*mm.*	
125	C.					57 7	65 5	7 23
138	"	20 53	17 71	86 3	50 0	52 5	65 5	7 19
139	"	20 53	14 23	69 3	33 5	45 1	41 5	7 33
153	"	20 53	15 35	74 8	37 0	54 4	52 0	7 31
164	"	18 83	14 25	75 7	37 5	53 1	53 0	7 29
167	"	16 00	9 70	60 6	28 5	54 5	56 0	7 28
171	"	18 03	9 06	50 2	24 0	58 4	67 0	7 23
127	K.					58 5	54 5	7 33
137	"	20 77	14 63	70 4	34 0	58 2	59 5	7 28
144	"	19 87	10 66	53 6	25 5	54 4	50 0	7 29
152	"	20 77	17 21	82 8	45 0	54 0	43 5	7 39
143	B.	19 30	16 03	83 0	45 0	47 0	44 0	7 32
157	D.	18 40	15 30	83 2	45 2	48 1	47 5	7 30
Average						53 5	53 6	7.29

the CO_2 curves were drawn are tabulated with the charts. A colorimetric determination of the pH of the dialysate of the venous blood was also made on each of these cases at the same time that the CO_2 and O_2 analyses were conducted. The colorimetric pH results and the essential clinical data are included in the preceding paper.[1] To avoid repetition and facilitate reference for clinical data, the division of cases into tables and the arrangement of the carcinoma cases within the tables according to increasing size and malignancy of the tumor is the same as that of the former paper.

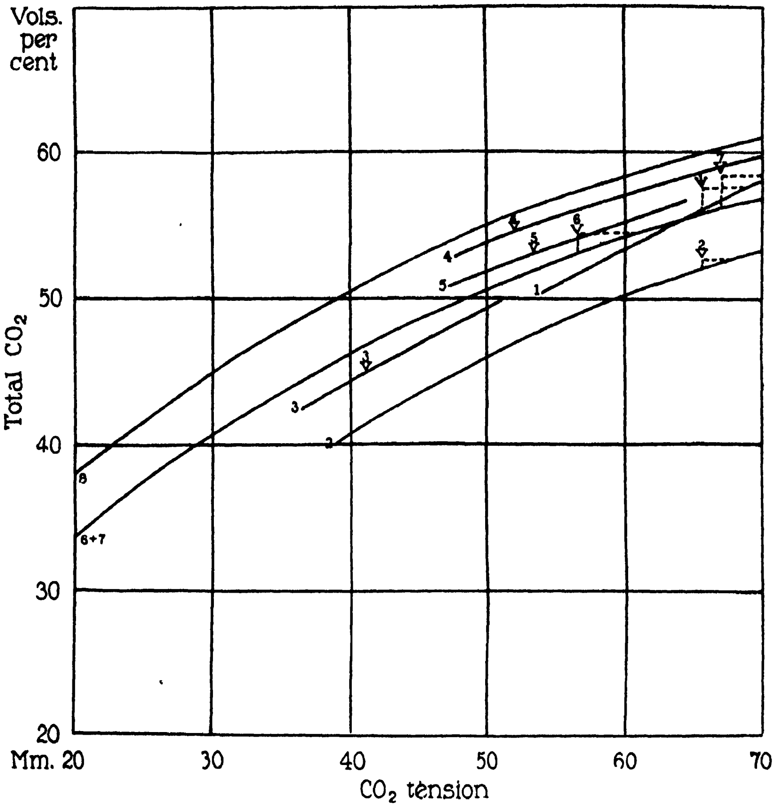

CHART 3. CO_2 absorption curves and venous points of the blood of normal Subject C.

Curve No.	Blood No.	O_2 tension.
		mm
1	125	140
2	138	82
3	139	31
4	153	36
5	164	35
6	167	660
7	171	585
8	167 171	7 15

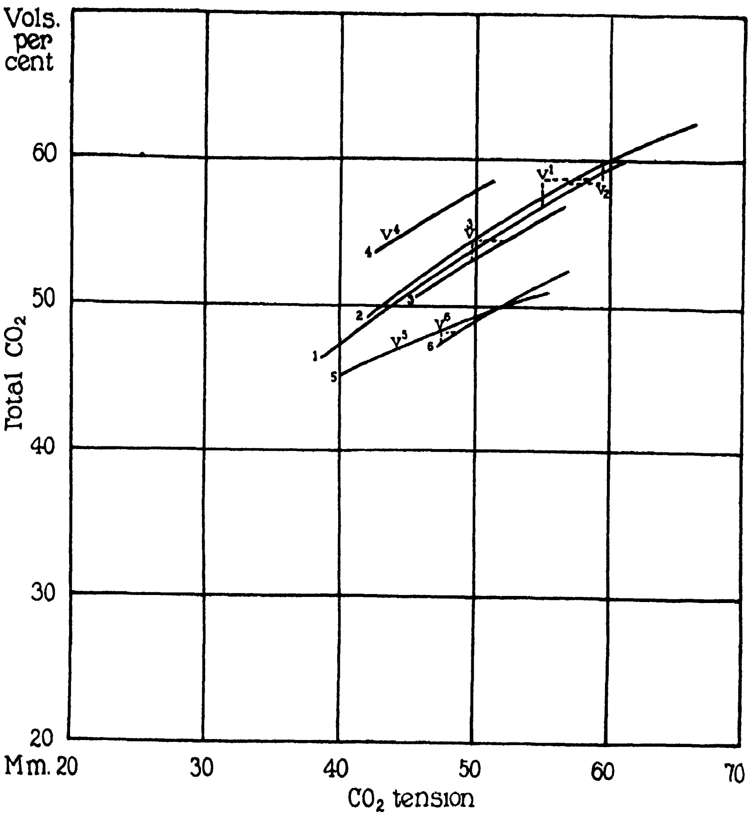

Chart 4. CO_2 absorption curves and venous points of the blood of normal Subjects K, B, and D.

Curve No.	Subject.	Blood No.	O_2 tension.
			mm.
1	K.	127	140
2	"	137	23
3	"	144	31
4	"	152	44
5	B.	143	45
6	D.	157	40

The blood numbers of the individual samples correspond in both publications.

The data for the normal bloods are contained in Table II, and the curves for Subject C, in Chart 3 and for Subjects K, B, and D, in Chart 4. Seven determinations were made at intervals over a period of 10 months on Subject C. All the points, with one exception, for the fully oxygenated blood of Samples 167 and 171 coincide to form the single curve numbered 6 + 7 in Chart 3. Curve 1, the other fully oxygenated one, agrees fairly well with it. The reduced curve from the same bloods (Nos. 167 and 171) was plotted (Curve 8) to illustrate the extreme variation in CO_2 content between fully oxygenated and almost completely reduced blood. Under constant conditions the curves for this blood when partially reduced, such as the venous blood sample, should lie between these two curves. Two of the curves, however, of partially reduced blood (Curves 2 and 3, Chart 3) fell below the fully oxygenated curves, or in other words showed a lowered amount of base available for combining with CO_2. An interval of 4 days separated these two occasions. The other five samples showed practically no variation in available base content for this individual. Similar variations in base content were found in the heights of the curves in the four determinations on Subject K (Curves 1 to 4). Probably this represents a local or temporary rather than a systemic or prolonged change in available base. It may have been caused by an increase in organic acids, a change in respiration, food ingestion, or excretion. The absorption curves agree in height and shape with the normal curves given by Peters, Barr, and Rule (1921) and those of Barcroft et al. (1922).

A variation in the individual was also found in both the CO_2 content and the CO_2 tension of the venous blood as shown in Table II. Several factors probably influence this difference in venous CO_2. The most obvious one, exercise, was controlled in all the subjects which were not confined to bed by a rest period of from 10 to 20 minutes. To what extent the activity or condition of the subject prior to the rest period or a change in the time of resting would influence the venous CO_2 tension has not been determined. The data on the normal subjects which are given in Table III and Chart 5 indicate that food ingestion is another possible factor in venous CO_2 variation.

The relation of the reduced to the oxygenated CO_2 absorption curves of these bloods has been shown in a previous publication discussing the buffers of the blood (Doisy, Briggs, Eaton, and Chambers, 1922). Our interest here lies in the pH of the venous blood of these normals as additional controls on the cancer cases. For the data given in Table III and Chart 5 the blood was drawn within 1 hour after breakfast, otherwise the rest period and imposed conditions were the same as those of all the other subjects. The venous CO_2 tension in every case was between 60 and 70 mm. with an average of 66.4 mm. (Table VII) or 12.8 mm. higher than the average of Table II. These two sets of normal blood were

TABLE III.

Oxygen, Carbon Dioxide, and pH of the Venous Blood at 38°C. of Normal Subjects.

Blood No.	Subject.	O_2 capacity of blood.	Chemically combined O_2 of venous blood.	O_2 unsaturation of venous blood.	CO_2 ordinate correction.	Venous CO_2 content.	Venous CO_2 tension.	pH
		vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*mm.*	
177	J. K.	20.55	12.80	7.75	2.09	60.0	70.0	7.22
182	"	22.33	9.17	13 16	3.55	60.7	67.0	7.25
174	M.	21.44	11.38	10 06	2.72	56.7	70.0	7.19
175	J. M.	19.57	11.81	7.76	2.10	53.9	61.0	7.23
178	M. D.	18.81	13.60	5.21	1.40	54 5	65.0	7.21
179	G. D.	21.10	10.38	10.72	2.90	57.6	65.5	7.23
Average						57.2	66 4	7.22

not essentially different in their absorption curves. The main difference was the high venous CO_2 tension of the bloods of Table III, with a corresponding increase in acidity, the average pH for Table III being 7.22 and for Table II being pH 7.29. The bloods of Table II were drawn between 2 and 4 hours after eating, so there seems to be a definite relation between the time elapsing after a meal and the venous CO_2 tension. In this connection the work of Dodds (1921) on the changes in alveolar CO_2 pressure following a meal is of particular interest. He found a rise in CO_2 tension of 2 to 6 mm. within 30 to 45 minutes after the meal, followed by a fall which lasted about 2 hours and a subsequent return to the original tension. From Dodd's work and the data presented in

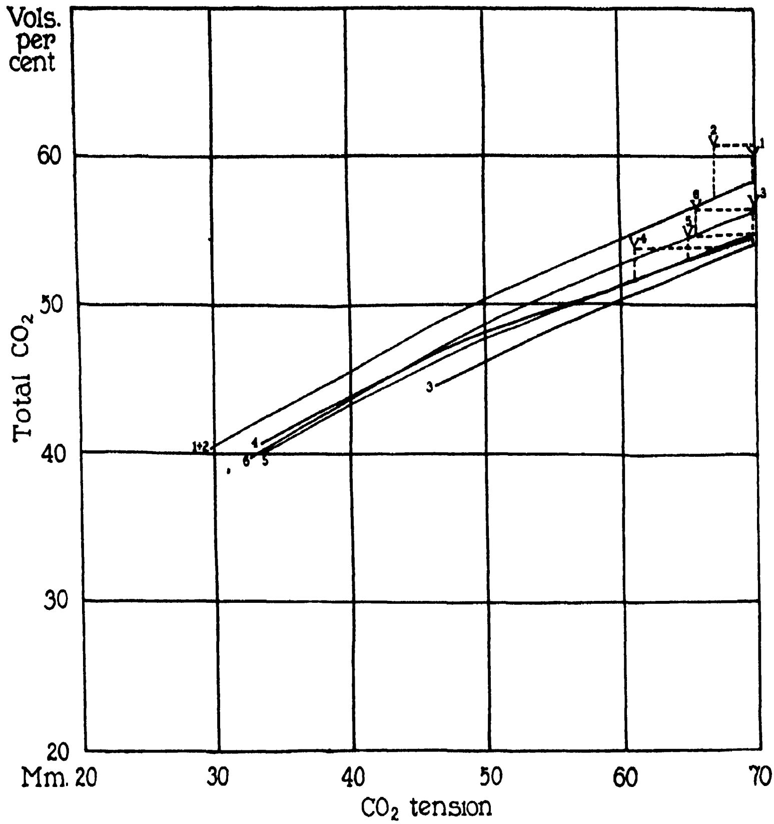

CHART 5. CO_2 absorption curves and venous points of the blood of normal subjects.

Curve No.	Blood No.	O_2 tension.
		mm.
1	177	140
2	182	140
3	174	139
4	175	139
5	178	138
6	179	140

Tables II and III, a correlation of the secretion of gastric juice with a variation in alveolar CO_2 tension, blood CO_2 tension, blood pH, and possibly the alkaline tide of the urine seems probable. If this is true, food ingestion is an important factor for consideration in determining the pH of the blood.

In calculating the average pH of the venous blood of the normal subjects for comparison with that of the carcinoma cases, the data of Table III were excluded because of the variation in the time elapsing after food ingestion. In the other normal subjects (Table II) and in all the other cases studied, this factor was more uniformly controlled, 2 to 4 hours being the time allowed after the meal before the blood was drawn, so the data were much more comparable. Table II shows some variation in pH in the different determinations on the same individual as well as between individuals. The average for each individual is as follows:

Subject.	No of determinations	Average pH of venous blood
C.	7	7.27
K.	4	7 32
B.	1	7.32
D.	1	7.30

The average of all the determinations on normal venous blood as shown in Table II was pH 7.29.

Diseases Other than Carcinoma.

As a further control on the carcinoma cases the bloods of several patients with other diseases were tested with the results shown in Table IV and Chart 6. The CO_2 absorption curves with the possible exception of the lowest one, Curve 4, fell within the normal limits of Charts 3 and 4. Most of these bloods had an oxygen capacity or hemoglobin content lower than normal (Table IV) which is reflected in a lower buffer action toward CO_2, making the curves approach the horizontal more nearly than the normal curves. The two determinations on the case of lues, Nos. 140 and 141, are of interest in their relation to the influence of food ingestion discussed in connection with Table III. Blood 140 with a venous CO_2 tension of 67 mm. was drawn about 1 hour

after breakfast, and Blood 141, with a venous CO_2 tension of 49 mm., was taken on the following day about 2 hours after lunch. Of the two cases of tuberculosis the one with the most extensive lung involvement, Blood 129, showed a low venous CO_2 tension, 32 mm., and a correspondingly low hydrogen ion concentration, pH 7.44. The average venous CO_2 content and CO_2 tension were slightly below the normal averages of Table II. Six of the eight pH determinations range between 7.29 and 7.37 with an average for all the cases in Table IV of pH 7.33.

TABLE IV.

Oxygen, Carbon Dioxide, and pH of the Venous Blood at 38°C. in Diseases Other than Carcinoma.

Blood No.	Diagnosis.	O_2 capacity of blood.	Chemically combined O_2 of venous blood.	Percentage saturation of venous blood.	Venous O_2 tension.	Venous CO_2 content.	Venous CO_2 tension.	pH
		vol. per cent	*vol. per cent*		*mm.*	*vol per cent*	*mm.*	
160	Fibroid uterus.	13.27	11 46	86 4	50.0	45.7	40 5	7.35
161	" "	19.87	7 10	35 7	19.0	54.5	46.2	7.36
163	Cervicitis.	14 40	3 30	22 9	15 0	55 5	47.4	7.37
140	Lues.*		5 36		21.0	50.1	67.0	7.16
141			7 50		37.0	48 6	49.0	7.29
145	Tuberculosis of rectum and lungs.	10 87	3 40	31.5	18.0	50.6	45.7	7.34
156		15.30	1.14	7.5	8.0	45.9	42.0	7.33
129	Tuberculosis of lungs.					43.8	32 0	7.44
Average						49 3	46.2	7.33

* Numbers enclosed in a bracket in all tables are separate determinations on different days on the same patient.

Carcinoma Cases.

The data on twenty-three blood samples from twenty patients with carcinoma are contained in Tables V, VI, and VII, and Charts 7, 8, and 9. The twenty-three CO_2 absorption curves showed in carcinoma no consistent change of base content available for combining with CO_2. The majority of the curves were normal in

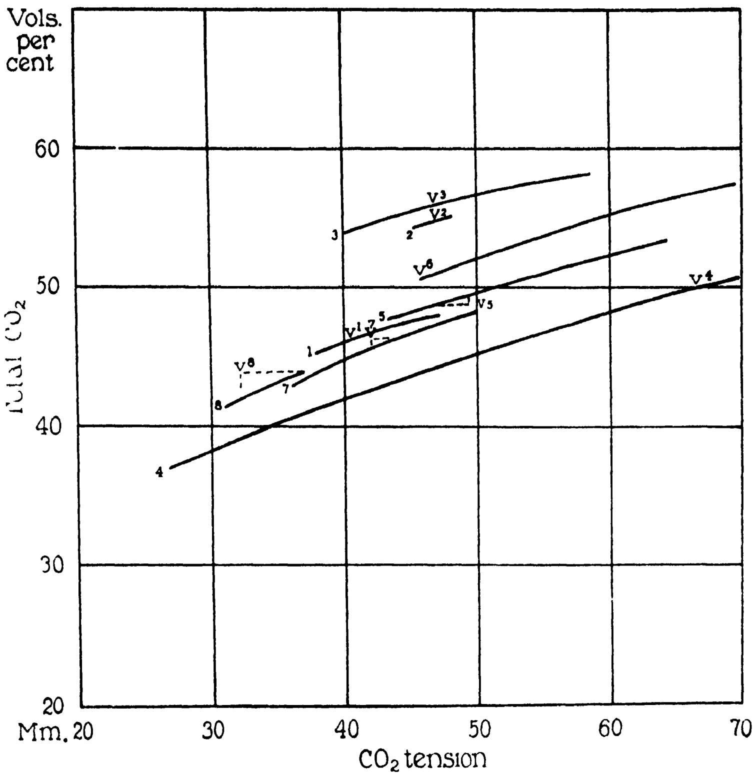

CHART 6. CO_2 absorption curves and venous points of the blood in diseases other than carcinoma.

Curve No.	Blood No.	O_2 tension.
		mm.
1	160	45.0
2	161	19.7
3	163	12.0
4	140	22.0
5	141	27.0
6	145	18.0
7	156	14.5
8	129	140.0

shape and within normal height limits. A few, however, were abnormally low, such as Curves 5 and 6 of Chart 7 and Curve 6 of Chart 8, and a few were high such as Curves 2 and 4 of Chart 8 and Curve 8 of Chart 9. A wide variation was also found in the venous CO_2 content and CO_2 tension, but with two exceptions the tension range was no greater than that found in the miscellaneous

TABLE V.

Oxygen, Carbon Dioxide, and pH of the Venous Blood at 38°C. in Carcinoma of the Breast.

Blood No.	Diagnosis.	O_2 capacity of blood.	Chemically combined O_2 of venous blood.	Percentage saturation of venous blood.	Venous O_2 tension.	Venous CO_2 content.	Venous CO_2 tension.	pH
		vol. per cent	*vol. per cent*		*mm.*	*vol. per cent*	*mm.*	
180	Carcinoma of right breast.	14.80	11.12	75.1	37.0	51.7	46.5	7.34
151 }	Carcinoma of left	13.03	6 56	50.4	24.0	53.8	51.5	7.31
154 }	breast and glands.	18.57	11.25	59.2	28.0	48.4	34.5	7.45
165	" " "	17.47	9 87	56.5	26.5	50.8	45.0	7.35
128	Carcinoma of right breast and glands.					43.4	46.5	7.26
115	Carcinoma of left breast and glands.					50.6	60.5	7.21
133	Carcinoma simplex.					57.1	58.0	7.29
113	Carcinoma of breast bilateral, skeleton, and pleura.					45.9	37.5	7.39
Average						50.2	48.8	7.31

diseases (Table IV). The averages of the venous CO_2 contents and tensions of these three tables compare favorably with those of Tables II and IV.

The hydrogen ion concentration of the blood of these carcinoma cases was not different from that of the normal bloods or the bloods from other diseases. Nineteen of the twenty-three blood samples reacted between pH 7.20 and 7.40; the other four were more alkaline, pH 7.44, 7.45, 7.46, and 7.63. The averages for the three

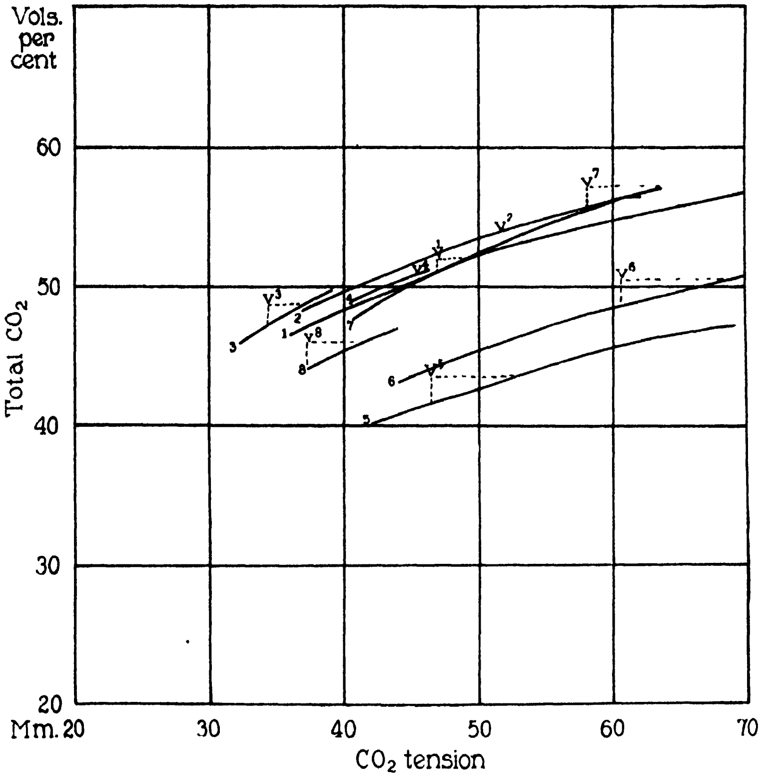

CHART 7. CO_2 absorption curves and venous points of the blood in carcinoma of the breast.

Curve No.	Blood No.	O_2 tension.
		mm.
1	180	137
2	151	28
3	154	44
4	165	25
5	128	140
6	115	140
7	133	140
8	113	140

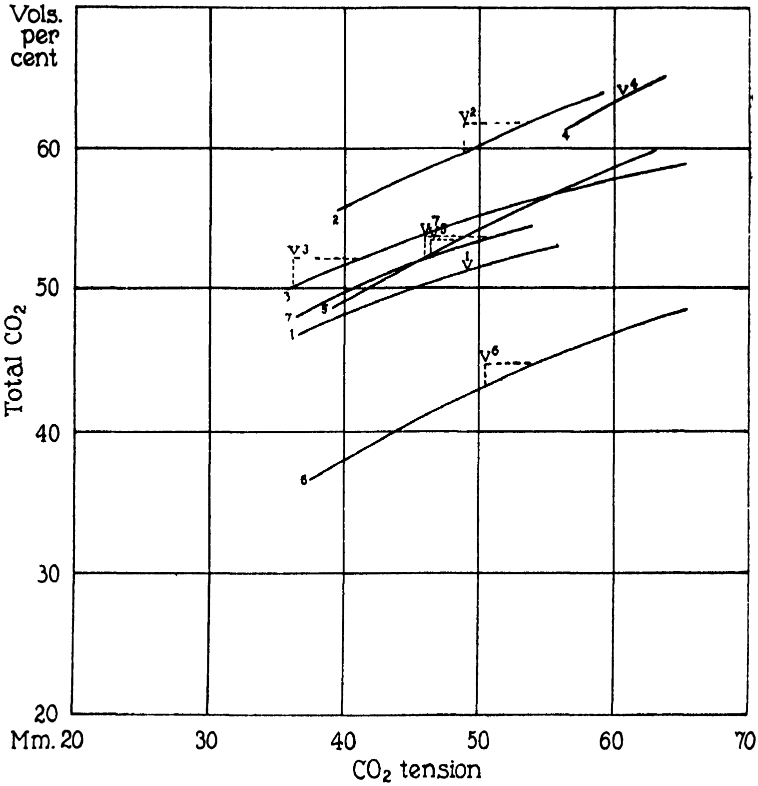

CHART 8. CO_2 absorption curves and venous points of the blood in carcinoma of the thoracic and abdominal organs.

Curve No.	Blood No.	O_2 tension.
		mm.
1	150	52
2	119	140
3	132	140
4	155	16
5	142	24
6	131	140
7	122	140

tables were pH 7.31, 7.35, and 7.35 as compared to pH 7.29 for Table II and pH 7.33 for Table IV. From the case descriptions tabulated in the preceding paper it is seen that the condition of the individual cancer patients differed widely. Some cases had only small tumors amenable to successful operation with no loss of weight, no anemia, or complicating pathology, while others had

TABLE VI.

Oxygen, Carbon Dioxide, and pH of the Venous Blood at 38°C. in Carcinoma of Thoracic and Abdominal Organs.

Blood No.	Diagnosis.	O_2 capacity of blood.	Chemically combined O_2 of venous blood.	Percentage saturation of venous blood.	Venous O_2 tension.	Venous CO_2 content.	Venous CO_2 tension.	pH
		vol per cent	*vol. per cent*		*mm.*	*vol. per cent*	*mm.*	
150	Carcinoma of sigmoid.		18.45		55.0	51.2	49.0	7.31
119	Carcinoma of rectum.					61.6	49.0	7.40
132	Carcinoma of pancreas and liver.					52.1	36.3	7.46
155		12.87	3.30	25.6	16.0	63.5	61.0	7.31
142	Carcinoma of rectum.		5.60		19.0	53 2	46.5	7.35
131	Carcinoma of stomach.					44.7	50.5	7.24
122	Carcinoma of lung, pleura, liver, and ovary.					53.7	46.0	7.36
Average						56.0	48 3	7.35

very extensive metastases with various degrees of cachexia, anemia, and organic diseases. For this reason it is not surprising that considerable variation was found in the CO_2 content, CO_2 tension, and reaction of the venous blood. The cases are arranged in the tables according to increasing size or malignancy of the tumor (see preceding paper[1]). No direct relationship between the reaction of the venous blood and the size of the tumor was indicated in the tables.

Comparing all of these data on normal, miscellaneous pathological, and cancer cases there is no evidence to show in the venous blood of patients with carcinoma an actual, uncompensated alkalosis which can be connected primarily with the presence of the tumor.

TABLE VII.

Oxygen, Carbon Dioxide, and pH of the Venous Blood at 38°C. in Carcinoma of the Pelvic Organs.

Blood No.	Diagnosis.	O_2 capacity of blood.	Chemically combined O_2 of venous blood.	Percentage saturation of venous blood.	Venous O_2 tension.	Venous CO_2 content.	Venous CO_2 tension.	pH
		vol. per cent	*vol. per cent*		*mm.*	*vol. per cent*	*mm.*	
173	Carcinoma of cervix.	11.48	6.54	57.0	27.0	57.3	70.0	7.20
121	Carcinoma of peritoneum.					45.1	32.5	7.44
162		19.20	6.60	34.4	18.5	56.5	44.8	7.40
130	Carcinoma of prostate.					52.5	61.8	7.22
126	Carcinoma of clitoris.					55.5	45.5	7.38
134	Inoperable carcinoma of cervix.					57.9	59.0	7.28
146	" " "	6.47	4 20	65.0	31.0	53.0	54.5	7.28
124	" " "					48.3	23.0	7.63
Average						53.3	48.9	7.35

DISCUSSION.

Summarizing the results presented in this paper and the preceding one of this series, we have found no definite change in the hydrogen ion concentration of the venous blood in cancer patients, but have observed an alkalosis in the dialysate of the same blood in the majority of the carcinoma cases. The difference in each case between the reaction of the venous blood and that of its dialysate is shown in Tables VIII to XII. The arrangement of the cases in the tables corresponds to that in the main data. In the normal

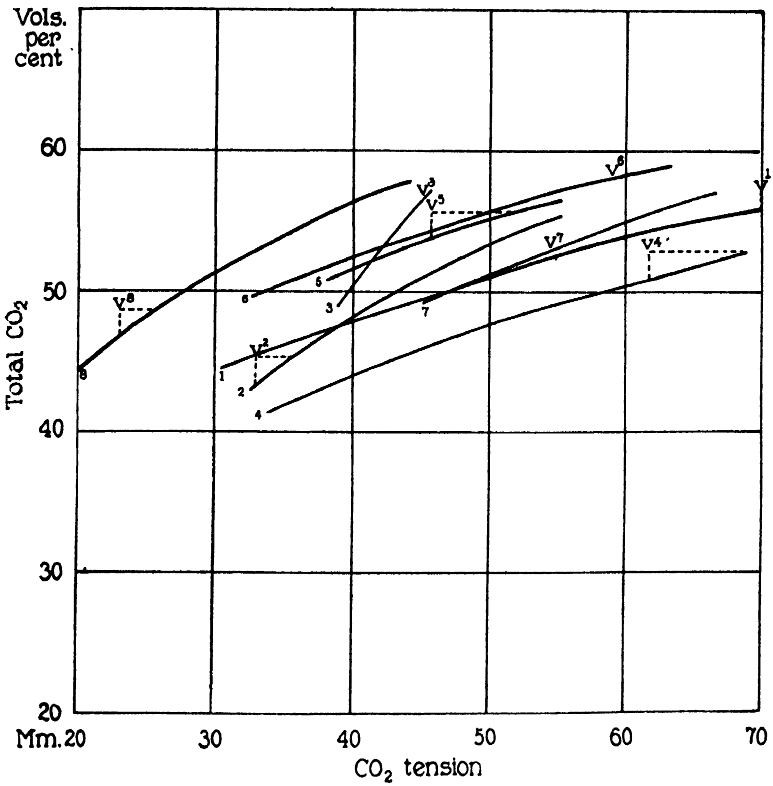

CHART 9. CO_2 absorption curves and venous points of the blood in carcinoma of the pelvic organs.

Curve No.	Blood No.	O_2 tension.
		mm.
1	173	143
2	121	140
3	162	20
4	130	140
5	126	140
6	134	26
7	146	24
8	124	140

subjects (Table VIII) and the miscellaneous pathological cases (Table IX) the agreement between the pH of the blood and that of its dialysate is very good. The difference is both positive and negative, but in the normals (Table VIII) the dialysate averages pH 0.03 higher than the blood and in the miscellaneous cases (Table IX) it averages pH 0.01 more alkaline. With the carcinoma cases the difference is greater, averaging pH 0.11 in carcinoma of the breast (Table X), pH 0.05 in carcinoma of thoracic and abdominal organs (Table XI), and pH 0.14 in carcinoma of the pelvic organs (Table XII). In most of the advanced cases

TABLE VIII.

Hydrogen Ion Concentration of the Venous Blood and Its Dialysate in Normal Subjects.

Blood No.	Subject.	Colorimetric pH of dialysate at 20°C.	pH from CO_2-bicarbonate ratio at 38°C.	Difference.
125	C.	7.43	7.23	+0.20
138	"	7.10	7.19	−0 09
139	"	7.28	7.33	−0.05
153	"	7.34	7.31	+0.03
164	"	7.33	7.29	+0 04
167	"	7.32	7.28	+0 04
127	K.	7.45	7.33	+0.12
137	"	7.21	7.28	−0.07
144	"	7.38	7.29	+0.09
152	"	7.36	7.39	−0.03
143	B.	7.39	7.32	+0.07
157	D.	7.30	7.30	0 00
Average....................		7.32	7.29	+0 03

of carcinoma the dialysate was pH 0.12 to 0.31 more alkaline than the blood.

In this comparison of the pH of the venous blood with the pH of its dialysate, it should be noted that the pH of the blood was calculated for 38°C. while the dialysate was tested at room temperature and reported at 20°C. To make these two sets of data comparable at 38°C. pH 0.18 must be added to the pH of the dialysate at 20° (according to the temperature correction factor in the preceding paper). This temperature correction was not made in these tables inasmuch as the agreement between the pH of the dialysate at 20° and the blood at 38° is very close in the

normal individuals and in the pathological cases other than carcinoma (Tables VIII and IX). In this way the difference in the carcinoma cases is emphasized.

TABLE IX.

Hydrogen Ion Concentration of the Venous Blood and Its Dialysate in Diseases Other than Carcinoma.

Blood No.	Diagnosis.	Colorimetric pH of dialysate at 20°C.	pH from CO_2-bicarbonate ratio at 38°C.	Difference.
160	Fibroid uterus.	7.33	7.35	−0.02
161	" "	7.35	7.36	−0.01
163	Cervicitis.	7.40	7.37	+0 03
140	Lues.	7.14	7.16	−0.02
141		7.27	7.29	−0.02
145	Tuberculosis of rectum and lungs.	7.43	7.34	+0.09
156		7.41	7.33	+0.08
129	Pulmonary tuberculosis.	7.40	7.44	−0 04
Average		7.34	7.33	+0.01

TABLE X.

Hydrogen Ion Concentration of the Venous Blood and Its Dialysate in Carcinoma of the Breast.

Blood No.	Diagnosis.	Colorimetric pH of dialysate at 20°C.	pH from CO_2-bicarbonate ratio at 38°C.	Difference.
180	Carcinoma of right breast.	7.26	7.34	−0.08
151	Carcinoma of left breast and glands.	7.32	7.31	+0.01
154		7.42	7.45	−0.03
165	" " " " " "	7.47	7.35	+0.12
128	" " right " " "	7.45	7.26	+0.19
115	" " left " " "	7.44	7.21	+0.23
133	" simplex.	7.51	7.29	+0.22
113	" of breast bilateral, skeleton, and pleura.	7.47	7.39	+0.08
Average		7.42	7.31	+0.11

It is concluded from the data of Tables VIII and IX that in normal individuals and in such pathological cases other than carcinoma as were studied, the pH of the dialysate at 20°C. agrees with the hydrogen ion concentration of the blood at 38°C. calculated

from the CO_2-bicarbonate ratio. The colorimetric determination by dialysis is the simpler method and more readily adapted to clinical application. The explanation of the difference of pH 0.18 between the blood at 38° and the dialysate at 38° and the explana-

TABLE XI.

Hydrogen Ion Concentration of the Venous Blood and Its Dialysate in Carcinoma of the Thoracic and Abdominal Organs.

Blood No.	Diagnosis.	Colorimetric pH of dialysate at 20°C.	pH from CO_2-bicarbonate ratio at 38°C.	Difference.
150	Carcinoma of sigmoid.	7.32	7.31	+0.01
119	" " rectum.	7.37	7.40	−0.04
132	" " pancreas and liver.	7.41	7.46	−0.05
155		7.35	7.31	+0.04
142	" " rectum.	7.34	7.35	−0.01
131	" " stomach.	7.53	7.24	+0.29
122	" " lung, pleura, liver, and ovary.	7.45	7.36	+0.09
Average		7.40	7.35	+0.05

TABLE XII.

Hydrogen Ion Concentration of the Venous Blood and Its Dialysate in Carcinoma of the Pelvic Organs.

Blood No.	Diagnosis.	Colorimetric pH of dialysate at 20°C.	pH from CO_2-bicarbonate ratio at 38°C.	Difference
173	Carcinoma of cervix.	7.43	7.20	+0.23
121	" " peritoneum.	7.55	7.44	+0.11
162		7.36	7.40	−0.04
130	" " prostate.	7.53	7.22	+0.31
126	" " clitoris.	7.58	7.38	+0.20
134	Inoperable carcinoma of cervix.	7.45	7.28	+0.17
146	" " " "	7.46	7.28	+0.18
124	" " " "	7.53	7.63	−0.10
Average		7.49	7.35	+0.14

tion of the alkalosis of the dialysate observed in carcinoma cases is sought in the theory of Donnan concerning the equilibrium established between two solutions separated by a membrane impermeable to one or more ions.

Donnan's Membrane Equilibrium.

The original work of Donnan and the recent applications of his theory in biology have been reviewed by Lewis (1920) and Loeb (1922). Barcroft, Hill, and coworkers (1922) and Warburg (1922) are the first to discuss the adaption of this theory to the equilibrium in blood between the red corpuscles and the plasma. The cell wall of the red blood corpuscle is the semipermeable membrane separating the two phases, cell contents and plasma. The former authors assume the permeability of the ions H^+, OH^-, HCO_3^-, and Cl^-, and the impermeability of the phosphate and basic ions.

From Donnan's theory then the

$$\frac{\text{Concentration of H ions in the plasma}}{\text{Concentration of H ions in the cell}} = \frac{\text{Concentration of basic ions in the cell}}{\text{Concentration of basic ions in the plasma}}$$

if the action of the phosphate which is comparatively small is disregarded. Therefore, any difference in non-diffusible basic ion concentration in the two phases is reflected in an inverse difference in diffusible H ion concentration.

In an extensive mathematical treatise on the theory of the Henderson-Hasselbalch equation Warburg (1922) discusses further complications encountered in applying Donnan's theory to blood. He reviews the literature up to 1921 on the permeability of the red corpuscle membrane and assumes that the cations do not diffuse through it. Factors such as the division of both the cells and plasma into a water phase and a protein phase and dissociation of the electrolytes are accounted for. The migration and redistribution through the membrane of the chlorine and bicarbonate ions when CO_2 is added to the blood are explained by Donnan's theory. Warburg also includes a chapter on the reaction of the red blood corpuscles and a discussion of the previous work on this subject. His experimental data are calculated from pH determinations on mixtures of serum with a cell fluid which was hemolyzed by freezing or with saponin. Using horse blood at room temperature the cells and serum were found to have the same reaction when that of the serum was between pH 6.50 and 6.88. Where the serum reaction was more alkaline than this, the cells were more acid than the serum. The more alkaline the serum the greater the difference. With the few determinations

at pH 6.50 the cells were more alkaline than the serum. If human blood at 38°C. agrees with these results, within the normal range the cells are pH 0.085 to 0.11 more acid than the serum.

More recent experimental work favors these assumptions regarding the permeability of the red blood corpuscle. Diffusion through the cell membrane of the ions H^+, OH^-, and HCO_3^- is unquestioned. Doisy and Eaton (1921) have demonstrated the quantitative passage of Cl ions between cells and plasma with changes in CO_2 tension, but found no migration of Na or K ions. Kramer and Tisdall (1922) reported that the corpuscles contain no appreciable amount of Na or Ca and that K represents practically all of their fixed base, while Na forms about 92 per cent of the fixed base of the serum. The molar concentration of the total basic radicles was 0.1585 in the serum and 0.1137 in the corpuscles, and the excess of basic over acid radicles was 0.0258 in the serum and 0.0213 in the corpuscles. According to the equation of Barcroft and Hill an excess of non-diffusible base in the plasma would cause an excess of H ions in the cells. This agrees with Warburg's results and with the experiments of Conway and Stephen (1922) who found that laked corpuscles were pH 0.13 more acid than the whole blood and concluded that the inside of the red blood corpuscle has a higher concentration of H ions than the surrounding plasma.

Evans (1921) found that the dialysis method gave for blood a pH 0.20 higher than the hydrogen electrode reaction and ascribed the difference to an error in the latter method when it is used with solutions containing CO_2. Cullen (Cullen, 1922; and Cullen and Hastings, 1922) has found no difference between the electrometric and colorimetric determinations on solutions containing CO_2 and phosphates where dialysis was not used. He suggests two errors in the dialysis method as employed; a loss of CO_2, and a difference in H ion concentration due to Donnan's membrane equilibrium.

In this experimental work the error due to the loss of CO_2 has been minimized by the technique described in the preceding article. The same difference, however, which Evans found between the dialysis method and the hydrogen electrode results, were found between the reaction of the dialysate and that of the blood calculated from the CO_2-bicarbonate ratio. The calculated pH and

the colorimetric pH agreed on the bicarbonate solution.[3] In Table VIII the reaction of the dialysate at 38°C. averaged pH 0.21 higher than that of the blood, and in Table IX the average was pH 0.19 higher. The explanation of this difference on the basis of Donnan's theory is illustrated in the following diagram. The diagram pictures the dialysis of whole blood through a celloidin sac as a double Donnan equilibrium in which the three phases, the red blood corpuscles, the plasma, and the dialysate, are separated by the two semipermeable membranes, the erythrocyte cell wall and the celloidin sac.

	Cell wall.		Celloidin sac	
Red blood cell.		*Plasma.*		*Dialysate.*
H^+	$\rightleftarrows$	H^+	$\rightleftarrows$	H^+
OH^-	$\rightleftarrows$	OH^-	$\rightleftarrows$	OH^-
Cl^-	$\rightleftarrows$	Cl^-	$\rightleftarrows$	Cl^-
HCO_3^-	$\rightleftarrows$	HCO_3^-	$\rightleftarrows$	HCO_3^-
HPO_4^-	?	HPO_4^-	$\rightleftarrows$	HPO_4^-
$H_2PO_4^-$	?	$H_2PO_4^-$	$\rightleftarrows$	$H_2PO_4^-$
		Na^+	$\rightarrow$	Na^+
K^+				
Hb^-				
HbO^-				
		Protein$^-$		
pH 7.17 to 7.21		pH 7.30		pH 7.50

Such a diagram must necessarily be only an imperfect representation of the general principles involved owing to a lack of exact knowledge as to much of the detail. Only the anions and cations which are considered of importance in the equilibrium are included as the constituents of the three phases. K^+ is used to represent the total dissociated base of the red blood cell and Na^+ that of the plasma inasmuch as these are the predominating bases in these two phases. Hb^- and HbO^- indicate the ionized hemoglobin and oxyhemoglobin in the corpuscle, and Protein$^-$, the protein ions and other non-diffusible anions in the plasma. These three protein ions are designated as anions because the reaction of the phases is to the best of our knowledge alkaline from their

[3] Chambers,[1] p. 235.

isoelectric points. Michaelis gives the following isoelectric points: serum albumin pH 4.70, serum globulin pH 5.40, and oxyhemoglobin pH 6.75. The free migration of the H^+, OH^-, Cl^-, and HCO_3^- ions between erythrocyte and plasma is shown in the diagram. Concerning the permeability of the cell wall for the phosphates the data are inconclusive, while no diffusion of the Na^+, K^+, Hb^-, HbO^-, and Protein$^-$ ions is indicated.

The equilibrium between erythrocyte and plasma is not as simple as were the systems constructed for experimental study by Donnan, Loeb, and others, who used only one non-diffusible ion on one side of the membrane. What effect the presence of several non-diffusible anions and cations on both sides of the membrane might have on the distribution of the diffusible ions cannot be stated. According to Hill, as discussed above, from the present available data the anions Hb^-, HbO^-, and Protein$^-$ are of minor importance in this equilibrium, and an excess of basic ion concentration in the plasma is a prime factor in increasing the relative H ion concentration in the cell. On the contrary the data of Warburg (1922) seem to indicate that the hemoglobin is significant in the equilibrium. His results, as noted above, showed a zone (pH 6.50 to 6.88) in which the serum and cells had the same reaction, with a positive difference on the alkaline side of this zone and a negative difference on the acid side. This is of interest in that the isoelectric point of oxyhemoglobin probably falls between pH 6.50 and 6.88 (pH 6.75), which is the point where the oxyhemoglobin ion would change its charge, being HbO^- on the alkaline side and HbO^+ on the acid side. Inasmuch as the hemoglobin is non-diffusible, the change from negative to positive may be the factor which causes the inverse distribution of the H ions at a pH of 6.50 and probably below. To illustrate in the diagram the difference in distribution of the H ions, arbitrary pH figures are given below it. Using an average reaction of pH 7.30 for the plasma, the reaction of the cells would be pH 7.17 according to Conway and Stephen (1922), and about pH 7.21 according to Warburg (1922).

The equilibrium between plasma and dialysate through the celloidin membrane is more typical of Donnan's original illustration in that all the non-diffusible ions (Protein$^-$ in the diagram) are on one side of the membrane, which is permeable to all the

crystalloidal ions. In applying Donnan's equations his designations are used; namely, brackets to indicate molar concentrations, inferior figure 1 for the plasma, and inferior figure 2 for the dialysate. From the diagram,

$$\frac{[Na^+]_1 + [H^+]_1}{[Na^+]_2 + [H^+]_2} = \frac{[OH^-]_2 + [Cl^-]_2 + [HCO_3^-]_2 + [HPO_4^-]_2 + [H_2PO_4^-]_2}{[OH^-]_1 + [Cl^-]_1 + [HCO_3^-]_1 + [HPO_4^-]_1 + [H_2PO_4^-]_1}$$

and

$$\frac{[Na^+]_1}{[Na^+]_2} = \frac{[H^+]_1}{[H^+]_2} = \frac{[OH^-]_2}{[OH^-]_1} = \frac{[Cl^-]_2}{[Cl^-]_1}$$

etc.

Since the total concentration of cations must equal that of the anions on each side of the membrane then

$$[Na^+]_1 + [H^+]_1 = [OH^-]_1 + [Cl^-]_1 + [HCO_3^-]_1 + [HPO_4^-]_1 + [H_2PO_4^-]_1 + [Protein^-]$$

and

$$[Na^+]_2 + [H^+]_2 = [OH^-]_2 + [Cl^-]_2 + [HCO_3^-]_2 + [HPO_4^-]_2 + [H_2PO_4^-]_2$$

Therefore

$$[Na^+]_1 + [H^+]_1 > [Na^+]_2 + [H^+]_2$$

and since

$$\frac{[Na^+]_1}{[Na^+]_2} = \frac{[H^+]_1}{[H^+]_2}$$

therefore the H ion concentration of the plasma, $[H^+]_1$, is greater than the H ion concentration of the dialysate, $[H^+]_2$. Such a distribution of H ions was found in this experimental work in the dialysis of blood from normal individuals and from miscellaneous pathological cases other than carcinoma. The average reaction of the dialysate was pH 0.20 higher than that of the blood. This difference is indicated by the figures below the diagram as pH 7.30 for the plasma and pH 7.50 for the dialysate.

In the bloods from the majority of the patients with carcinoma the pH of the dialysate was more alkaline than that of the normals. As the combined data of Tables X, XI, and XII show the degree of alkalinity varied from the normal by an average of pH 0.08, with a variation up to pH 0.31 in some of the more advanced cases. In other words, using the same figure of pH 7.30 for

plasma, the cancer cases would average pH 7.58 for the dialysate instead of the normal pH of 7.50 and some of the cancer cases would have a pH between 7.70 and 7.81. The results of the CO_2-bicarbonate ratio calculations showed no change from the normal in the concentration of H ions in the plasma in cancer. For simplicity in applying Donnan's theory to explain this increase in the alkalinity of the dialysate, symbols are substituted for the radicals as follows:

Let $X = [Na^+]_1 + [H^+]_1$

then $X = [OH^-]_1 + [Cl^-]_1 + [HCO_3^-]_1 + [HPO_4^=]_1 + [H_2PO_4^-]_1 + [Protein^-]_1$

and if $Z = [Protein^-]_1$

$X - Z = [OH^-]_1 + [Cl^-]_1 + [HCO_3^-]_1 + [HPO_4^=]_1 + [H_2PO_4^-]_1$

Let $Y = [Na^+]_2 + [H^+]_2$

then $Y = [OH^-]_2 + [Cl^-]_2 + [HCO_3^-]_2 + [HPO_4^=]_2 + [H_2PO_4^-]_2$

From Donnan's equation, $X (X - Z) = Y^2$, or $\frac{X}{Y} = \frac{Y}{X - Z}$.
A reduction of H ion concentration in the dialysate and no change in H ion concentration in the plasma is expressed in this equation as a decrease in the value of Y and no change in the value of X. With such a change in the valuation of Y the equilibrium of the equation can be maintained only by an increase in the value of Z, which represents the concentration of the non-diffusible anions in the plasma. Concerning any changes in the total basic ion concentration in cancer, nothing can be deduced from these equations except that the distribution of basic ions between the plasma and dialysate conforms to that of the H ions according to the equation $\frac{[Na^+]_1}{[Na^+]_2} = \frac{[H^+]_1}{[H^+]_2}$.

For the difference in hydrogen ion concentration between normal whole blood and its dialysate, found in the experimental data, Donnan's membrane equilibrium theory seems to offer a logical explanation. It also indicates that the alkalinity of the dialysate noted in the advanced cases of carcinoma was due to an increase of non-diffusible anions in the plasma.

SUMMARY.

The CO_2 content and the CO_2 tension were determined in the venous blood from normal individuals, from patients with carcinoma, and from miscellaneous pathological cases other than carcinoma. The venous CO_2 tensions were read from the CO_2 absorption curves which were plotted at the venous oxygen tension, or from those plotted from fully oxygenated blood with a correction for venous oxygen unsaturation.

The hydrogen ion concentration of the blood was calculated from the CO_2-bicarbonate ratio. The average reaction of twelve determinations on normal bloods was pH 7.29, of eight determinations on diseases other than carcinoma it was pH 7.33, and of twenty-three determinations on carcinoma patients it was pH 7.34.

It is concluded that these results furnish no evidence of an actual decrease in H ion concentration of the blood in carcinoma which can be related primarily to the cancer.

In normal bloods the colorimetric determination of the pH of the dialysate at 20°C. agreed within experimental error with the pH of the venous blood at 38°C., calculated from the CO_2-bicarbonate ratio.

An illustration is presented of the distribution of the ions in the three phases, corpuscle, plasma, and dialysate, according to Donnan's membrane equilibrium theory when whole blood is dialyzed through a celloidin sac. The theory accounts for the difference in pH between the plasma and dialysate in normal blood and for the greater concentration of H ions in the plasma.

The alkalosis of the dialysate in carcinoma cases compared to normal blood is explained by Donnan's theory as due to an increase in non-diffusible anions in the plasma.

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STUDIES ON CARBOHYDRATE METABOLISM.*

I. SOME COMPARISONS OF BLOOD SUGAR CONCENTRATIONS IN VENOUS BLOOD AND IN FINGER BLOOD.

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(Received for publication, December 5, 1922.)

In the course of an investigation of certain phases of carbohydrate metabolism it became desirable to compare the concentrations of sugar in arterial and in venous blood during active sugar absorption. Ege and Henriques (1) have recently reported a study of the sugar content of venous and arterial blood of dogs under various conditions, but their paper contains no data bearing on the point we had in mind.

A few attempts soon convinced us that we could not take serial samples from the radial arteries of our human subjects. As a substitute for pure arterial blood we have investigated the sugar content of blood obtained by lancing the finger tip. "Finger blood" has been used to a great extent in studies of blood sugar, especially by European workers, but we are unable to find record of any extensive comparison of it with either pure arterial or venous blood. In order to judge how nearly the finger blood approximates pure arterial blood the following experiments were performed. A dog was given 50 gm. of glucose in solution by mouth; half an hour later, under novocaine and light ether anesthesia, samples were taken as nearly simultaneously as possible from the femoral artery, the femoral vein, and the pad of the hind foot, by lancing. The three samples were obtained within a period of 5 minutes. Analyses for sugar by the Folin-Wu method gave the following results.

	mg per cc
Femoral vein	176
Femoral artery	210
Foot	208

* Aided by a grant from the Research Board of the University.

In another experiment a dog was anesthetized with urethane and novocaine. 50 gm. of glucose were given by stomach tube. An hour later simultaneous samples from the jugular vein, the carotid artery, and the foot gave results as follows:

	mg per 100 cc.
Jugular vein	184
Carotid artery	208
Foot	203

From the above data it is evident that, with regard to its sugar content, the blood obtained by lancing the foot is practically identical with arterial blood, and we conclude that the sugar values which are found in finger blood are substantially the same as would be found on analysis of pure arterial specimens.

Methods.

The experimental subjects were healthy students (men and women) who had become familiar with the technique of venous puncture both as subjects and as operators. The experiments were carried out in the morning, the subjects having omitted breakfast. The sugars used were all of high purity. The glucose was Pfanstiehl's C.P. Special, the galactose was partly Pfanstiehl's best grade and partly prepared in this laboratory by Mr. E. McKay according to E. P. Clark's (2) excellent method, the product being apparently equal to the best on the market. The fructose was Pfanstiehl's "pure" grade. The sugars were ingested in approximately 20 per cent solution immediately after preliminary blood samples had been taken. Whole blood was used, specimens being taken from an arm vein and from a finger tip. The Folin and Wu (3) method of analysis was employed. For the finger blood it was adapted to the use of a 0.3 cc. blood sample and 1 cc. of filtrate, using a constricted tube just half as large as the regular size. The micro adaptation is nearly as accurate as the original. In using the Folin-Wu method we have found it desirable to use a third standard solution containing 0.3 mg. of glucose per 2 cc.

A word might be added concerning the method of obtaining the finger blood. The hand if cold is immersed for a minute in warm water. The tip of a finger is then lanced with a spring lancet and the blood is collected directly into a pipette. The lancet is

ground to a chisel-like cutting edge 1 mm. wide and is set to deliver a prick about 3 mm. deep. No stasis is used but sometimes the finger is gently "milked" if the flow is not sufficiently rapid.

DISCUSSION.

From the results of our experiments with the ingestion of various carbohydrates (shown in Tables I to V and Charts 1 to 7) it appears that:

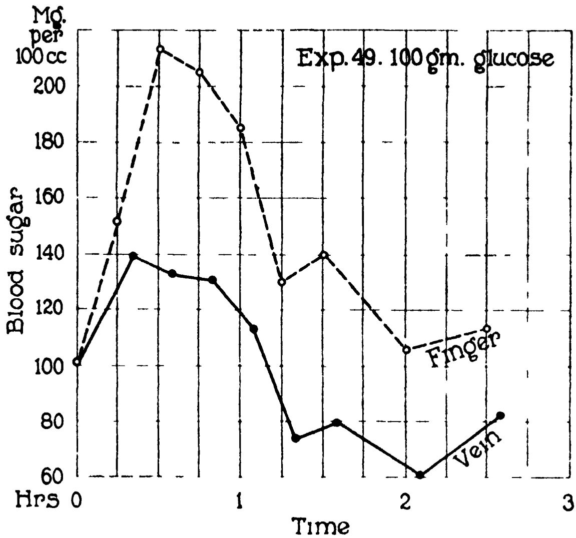

CHART 1. Sugar of venous and finger blood after ingestion of glucose. See Table I.

1. In the fasting condition the sugar concentrations of finger blood and venous blood are the same, or at least the difference is within the experimental error.

2. After the ingestion of glucose (Tables I and II, Charts 1 and 2) the sugar in the finger blood rises to much higher levels than in the venous blood.[1] For the first few minutes the curves are close together, the two lines then diverge, the rise in the venous blood being checked sooner and at a lower level. For a

[1] The lower values in the venous blood are not due to dilution of the blood as it passes through the tissues for we found no detectable difference between the water content of venous and finger blood during the glucose curve.

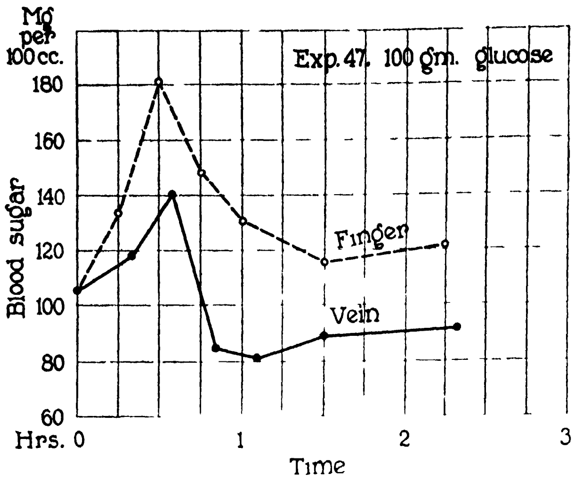

CHART 2. Sugar of venous and finger blood after ingestion of glucose. See Table I.

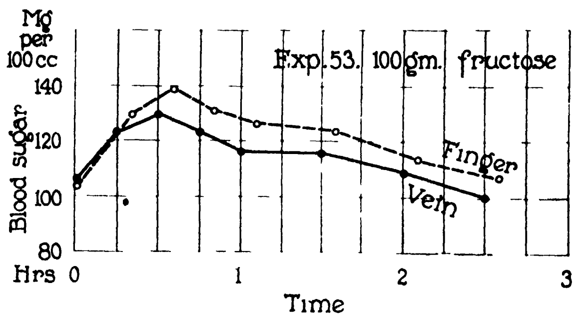

CHART 3. Sugar of venous and finger blood after ingestion of fructose. See Table III.

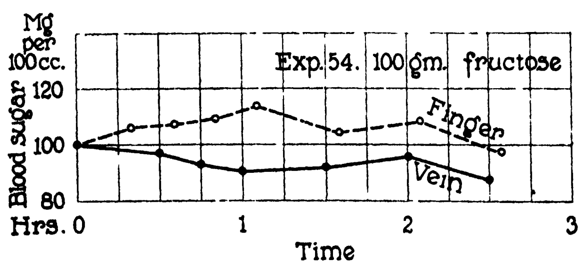

CHART 4. Sugar of venous and finger blood after ingestion of fructose. See Table III.

while the curves are roughly parallel, though at widely different levels, finally approaching each other again at normal or hypoglycemic levels. The hypoglycemia which nearly always follows the rise is much more marked in the venous than in the finger blood. In fact it is often missing in the latter. Our glucose experiments seem to indicate that the muscle tissues are more important

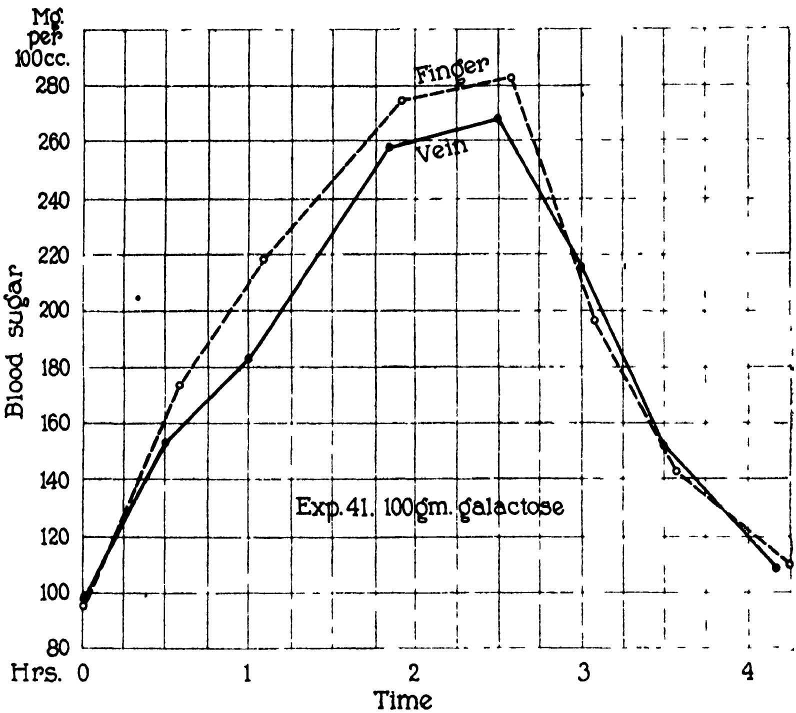

CHART 5. Sugar of venous and finger blood after ingestion of galactose. See Table IV.

in connection with glycogen storage than is perhaps generally believed (*cf.* McLean and de Wesselow (4)).

3. After taking fructose (see Table III, Charts 3 and 4) the rise in the finger blood is much less than after the ingestion of glucose. The rise in venous blood is slight and of short duration and is more often entirely missing. Apparently fructose is much more completely stopped by the liver than is glucose. This is interesting in view of the recent hint that the glucose of which

glycogen is composed is, in part at least, in the ethylene oxide form (5). Fructose is known to assume such a structure to a much greater extent than the aldohexoses. After the ingestion of fructose we have never observed the marked hypoglycemia in venous blood which is so often seen after taking glucose.

4. The curves obtained with galactose (see Table IV, Charts 5 and 6) show two points of interest. First the much higher levels of hyperglycemia, and secondly, the close correspondence of finger and venous blood throughout the curve. These obser-

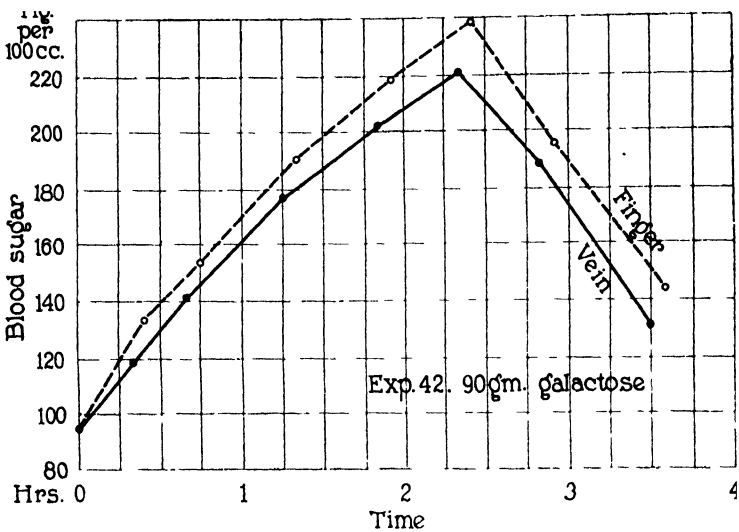

CHART 6. Sugar of venous and finger blood after ingestion of galactose. See Table IV.

vations fit in well with the known fact that galactose is a poor glycogen former. It is not readily retained by glycogen synthesis in the liver, hence the excessive hyperglycemia in the arterial (finger) blood, nor is there a marked glycogen formation in the muscles as indicated by the fact that the concentration in the venous blood is only slightly lower than in the finger blood samples.

The experiments with starch (Table V, Chart 7) were performed because of the recent results of Folin and Berglund (6), who reported no rise in the sugar content of venous blood after the

ingestion of starch. Jacobsen (7), Bang (8), and McLean and de Wesselow (4) had previously reported hyperglycemia in finger blood after feeding starch, their curves closely resembling glucose curves. Our results agree with those of the last mentioned group. The curves are quite similar to many of our glucose curves.

The sham feeding with agar-agar (Table V) was included because of the paper of Cammidge, Forsyth, and Howard (9), who obtained hyperglycemia after the ingestion of non-carbohydrate meals, even a cup of tea sufficing to raise the blood sugar level. Their conclusion was that the alimentary hyperglycemias have little

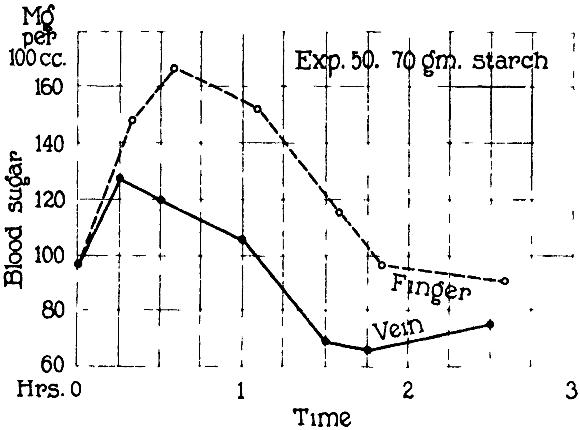

CHART 7. Sugar of venous and finger blood after ingestion of boiled starch. See Table V.

to do with the absorption of sugar from the gut, but are to be connected with acid-base changes due to secretion of gastric juice. We found no change in the sugar concentration of either venous or finger blood after the ingestion of agar.

Finally it might be well to point out that frequently repeated venous punctures and rather deep finger pricks called forth no indications of emotional hyperglycemia in our subjects. This was suggested by Folin and Berglund as a probable explanation of the generally higher levels of hyperglycemia found by those who have used finger blood. We have one observation which suggests that there is perhaps no such thing as a mild degree of emotional

hyperglycemia. During an unsuccessful attempt to draw a sample from the radial artery the needle was kept inserted for a rather long time and pushed hither and thither while trying to enter the artery. The pain of this operation is distinctly greater than from even a poorly executed venous puncture. The subject became very pale and was on the verge of fainting when the needle was withdrawn. Samples taken 40 minutes later showed 99 mg. in the venous and 98 in the finger blood. Before the attempt on the radial artery the values were 97 and 96, respectively. This, of course, proves nothing, but if this fairly strong stimulus failed to produce even a mild hyperglycemia it seems doubtful that a few needle pricks could have any effect at all.

TABLE I.

Sugar in Venous and Finger Blood after Ingestion of Glucose.

Time after ingestion.*	Blood sugar per 100 cc.							
	Experiment 39. 100 gm. glucose.		Experiment 49. 100 gm. glucose.		Experiment 47. 100 gm. glucose.		Experiment 43. 200 gm. glucose.	
	Vein.	Finger.	Vein.	Finger.	Vein.	Finger.	Vein.	Finger.
min.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Before.	105	104	101	102	105	105	96	94
15	157	170	140	152	118	134	149	174
30			133	214	141	182		
35	165	204						
40							83	129
45			131	205	85	148		
60	109	190	114	185	81	131		
75			74	130			81	110
90			80	140	89	116		
105	84	138					87	112
120			61	106	91	122		
150	68	87	82	114			81	109
170							79	113
190	90	97						
210							82	104

* Finger blood samples taken at the times noted, venous samples taken 5 minutes later in each case.

TABLE II.

Sugar Content of Venous and Finger Blood after Ingestion of Glucose.

Time after ingestion.*	Blood sugar per 100 cc.					
	Experiment 31. 100 gm. glucose.		Experiment 44. 200 gm. glucose.		Experiment 55. 200 gm. glucose.	
	Vein.	Finger.	Vein.	Finger.	Vein.	Finger.
min.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Before.	93	97	100	98	105	104
15			154	180	170	192
30	144	171	137	179	151	193
45	157	201			118	156
60					120	148
75	111	139	114	159		
90					115	149
105	83	116	96	124		
130			104	133	105	143
150					101	118
210					96	99

* Finger blood samples taken at the times noted, venous samples taken 5 minutes later in each case.

TABLE III.

Sugar Content of Venous and Finger Blood after Ingestion of 100 Gm. of Fructose.

Time after ingestion.*	Blood sugar per 100 cc.							
	Experiment 38		Experiment 53.		Experiment 54.		Experiment 37.	
	Vein.	Finger.	Vein.	Finger.	Vein.	Finger.	Vein.	Finger
min.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Before.	96	97	105	103	102	102	87	93
10							98	118
15	104	125	123	125		106		
20							84	116
30	99	122	130	139	97	107	76	121
45		123	123	132	93	109	74	107
60	101	121	116	125	91	114	79	112
90	105	113	115	124	92	104	79	107
120			109	114	96	109		
150			100	108	87	98	84	109

* Venous samples taken at times noted, finger blood samples 5 minutes later in each case.

TABLE IV.

Sugar Content of Venous and Finger Blood after Ingestion of Galactose.

Time after ingestion.*	Blood sugar per 100 cc.					
	Experiment 40. 80 gm. galactose.		Experiment 41. 100 gm. galactose.		Experiment 42. 90 gm. galactose.	
	Vein.	Finger.	Vein.	Finger.	Vein.	Finger.
min.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Before.	93	95	97	96	94	96
20	111	118			118	134
30			153	174		
40					142	154
50	130	142				
60			182	218		
75	161	175			177	190
110	192	212	258	276	202	218
140	203	212			221	238
150			269	284		
170	180	188			188	195
180			215	197		
210			152	143	131	144
250			109	110		

* Venous samples taken at times noted, finger blood samples 5 minutes later in each case.

TABLE V.

Sugar Content of Venous and Finger Blood after Ingestion of Starch and of Agar-Agar.

Time after ingestion.*	Blood sugar per 100 cc.					
	Experiment 50. 70 gm. starch.		Experiment 58. 100 gm. starch.		Experiment 51. 10 gm. agar-agar.	
	Vein.	Finger.	Vein.	Finger.	Vein.	Finger.
min.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Before.	96	95	93	100	106	107
15	128	148	112	143	105	105
30	120	166	118	151	102	104
45						
60	107	152	94	140	100	102
90	70	116	84	125	104	103
105	67	96			99	102
120	76	91	86	119		
150						

* Venous samples taken at times noted, finger blood samples 5 minutes later in each case.

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STUDIES ON CARBOHYDRATE METABOLISM.

II. AN INTERPRETATION OF THE BLOOD SUGAR PHENOMENA FOLLOWING THE INGESTION OF GLUCOSE.

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(Received for publication, December 5, 1922.)

The main facts regarding the blood sugar changes in response to the ingestion of glucose by normal man are well established. The sharp rise to a maximum followed by a rather rapid return to normal and often to markedly hypoglycemic levels has been shown by Jacobsen (1) and by many others. Obviously, the rise and fall are dependent on the relative velocities of the inflow of glucose from the intestine and its subsequent removal from the blood stream by the tissues. It is not surprising that when a large amount of readily diffusible glucose is suddenly introduced into the alimentary tract the rate of absorption should exceed the rate at which the tissues can abstract it from the blood. But it is not so clear why the curve should again fall to normal as rapidly as it often does at a time when the rate of absorption from the gut can be scarcely diminished. (Johansson (2) has evidence based on x-ray examinations that the absorption of 200 gm. of glucose is not complete until after about 4 hours, whereas we have often seen the concentration of sugar in the venous blood rise and return again to normal within ½ hour after ingesting the glucose.)

Nor is it clear why the curve should fall far below even the normal fasting level as it often does within an hour or more after taking glucose (*i.e.* at a time when there may be still considerable absorption taking place from the gut).

There have been some attempts to account for this remarkable increase in the rate of removal of glucose from the blood stream. Hypoglycemia after ingestion of glucose was demonstrated by

Liefmann and Stern (3) in 1906. In 1910 Frank (4) suggested that this hypoglycemia is due to overstimulation of the glycogen-forming function of the liver.

Recently McLean and de Wesselow (5) have put forth the same hypothesis. They present a comparison of the blood sugar curves of a normal man and of a severe diabetic after each had taken 50 gm. of glucose. The curves both rise sharply by nearly the same amount for the first half hour. At this point the normal curve breaks and soon begins to fall rapidly to the fasting level, while the curve of the diabetic continues to rise unchecked for 2 hours or more (*i.e.* presumably as long as absorption continues). Apparently, in the normal, some mechanism which was dormant for the first half hour has been awakened and intervenes to check the rising hyperglycemia. These authors argue that the presence of an excess of sugar in the circulation stimulates the mechanism which deals with sugar. They assume the mechanism to be glycogen synthesis, largely in the liver. The period of hypoglycemia in the normal curve they represent as being due to an "overactivity" of the awakened glycogen-forming function. In the severe diabetic glycogen synthesis does not occur appreciably and the hyperglycemia is excessive.

Our own results are wholly in accord with such a theory, and it is the purpose of this paper to present data which may strengthen and extend this view-point.

Methods.

The subjects for these experiments were students at this laboratory. The methods employed were the same as those outlined in the preceding paper.

Experiments.

First we would present a comparison analogous to McLean and de Wesselow's curves of normal and diabetic; namely, a comparison of the curves obtained after the ingestion of glucose and of galactose by normal man. (Charts 1 and 2. Table I.) In each case the comparison is made of a glucose curve and of a galactose curve of one individual. All of the curves presented are, according to our experience, typical normal reactions. The case seems to be entirely analogous to the above cited one of

McLean and de Wesselow. After the preliminary latent period there occurs an active glycogen synthesis from the glucose, and the hyperglycemia is rapidly reduced. Galactose, on the other hand, as is well known, is a poor glycogen former and the hyperglycemia rises unchecked for a much longer time.

We next present our results on the effect of repeated doses of glucose. (Tables II and III. Charts 3 and 4.) It will be recalled that in this connection McLean and de Wesselow raised a rather serious objection to their own hypothesis. If it is true

TABLE I

Sugar Content of Venous Blood after Ingestion of Glucose and of Galactose.

Time after ingestion.	Blood sugar per 100 cc.			
	Subject 1.		Subject 2.	
	Glucose (Experiment 1)	Galactose (Experiment 8)	Glucose (Experiment 2)	Galactose (Experiment 9).
min.	*mg.*	*mg.*	*mg.*	*mg.*
Before.	90	82	89	86
15		139		101
30	121	160	128	102
40				
45		170		123
60	132		77	
75		235		179
105		263		206
120	77		81	
135		290		247
180	94	186	89	161
240	89		103	

that the ingestion of glucose leads to an overstimulation of the glycogenic function, then a second dose of glucose should be more efficiently dealt with and should have less effect on the blood sugar level than the first. As a matter of fact, however, McLean and de Wesselow, analyzing finger blood, found that the second dose, if given soon after the curve had returned to normal caused a second rise almost identical with the first.

In our experience such a result is the exception rather than the rule. In our first experiments with repeated doses we analyzed only the venous blood. (Table II. Charts 3 and 4.) Some indi-

vidual variation was found, but in the majority of experiments (9 out of 13) the second dose of glucose, if taken after the curve had returned to normal or hypoglycemic levels, did not influence the venous blood sugar. In a few cases a second rise occurred but

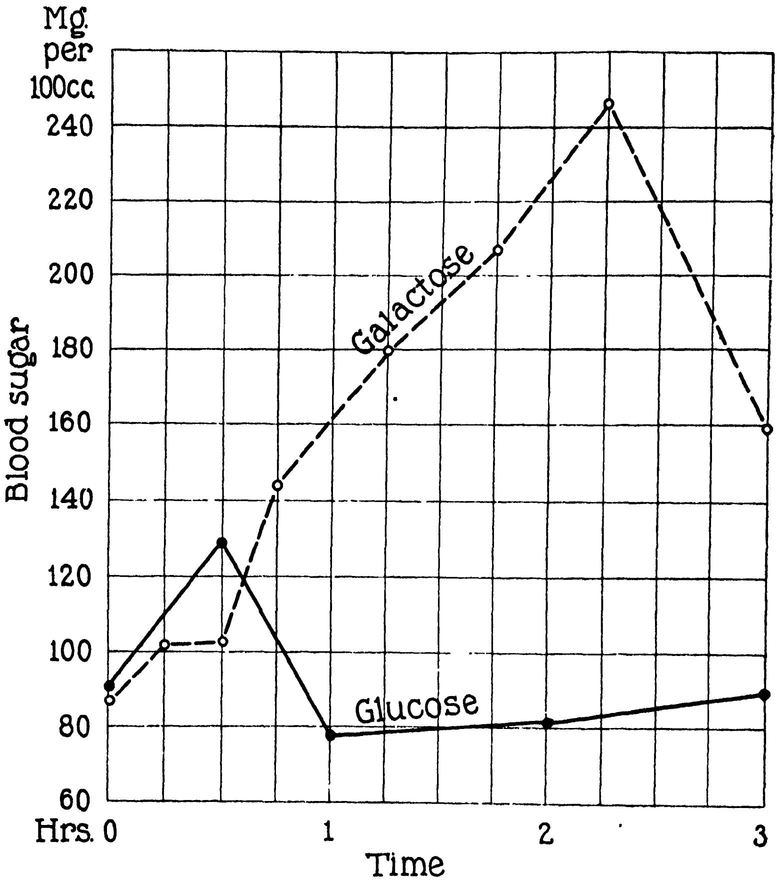

CHART 1. Contrasting curves of venous blood sugar after glucose and after galactose. (See Table I.)

it never was of the same magnitude as the first. In later experiments we analyzed both venous and finger (arterial) blood, and again in the majority of cases we found that the second dose exercised little or no effect on the blood sugar. (Table III. Chart 5.) In two cases, however, we obtained results similar

to those of McLean and de Wesselow. In these two experiments (one of which is illustrated by Chart 6) the finger blood showed a

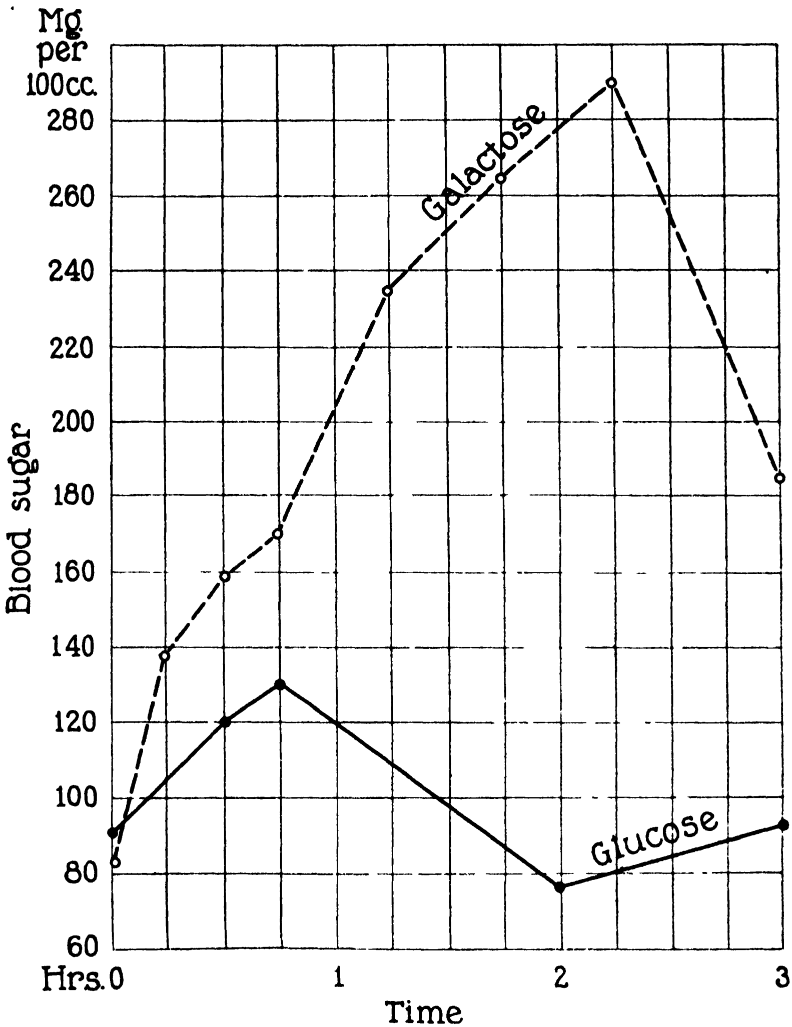

CHART 2. Contrasting curves of venous blood sugar after glucose and after galactose. (See Table I.)

second rise almost equal to the first. The venous blood sugar, however, rose only slightly after the second dose.

TABLE II.

Showing that a Second Dose of Glucose May Have Little or No Effect on the Sugar of Venous Blood.

Two doses of 100 gm. each. The second dose taken at time noted by asterisk (*).

Time after first dose.	Blood sugar per 100 cc.							
	Experiment 21.	Experiment 24.	Experiment 28.	Experiment 33.	Experiment 35.	Experiment 36.	Experiment 34.	Experiment 32.
min.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Before.	95	99	83	102	88	98	102	82
15	171	139	138	146	149	115	130	108
30	140	81	111	118	130	132		146
45	102					100	72	120
60	105*	62*	72	92	114			
75	101	57			101	83*	62	114
90	95	63	69*	94*	80*	98	*	87*
105	86	57	71	98	109	98	102	79
120			70	85	105	98	70	113
135		72		81	101		75	99
150			80					

* Second dose swallowed immediately after this blood sample was drawn.

TABLE III.

Sugar Content of Venous and of Finger Blood after Repeated Doses of Glucose.

Two doses of 100 gm. each. The second dose taken at time noted by asterisk (*).

Time after first dose.†	Blood sugar per 100 cc.					
	Experiment 45.		Experiment 59.		Experiment 46.	
	Vein.	Finger.	Vein.	Finger.	Vein.	Finger.
min.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Before.	96	102	104	107	110	109
15	131	160	124	137	143	176
30	151	198	111	160	154	196
45	123	188			128	172
60	105	162	93	143	105	158
75						
90	109*	137			102*	137
105	102	141	89*	136	120	180
120	105	134	93	140	114	160
135	102	139	93	144		
150	98	124	81	118	85	141
165					77	132

* Second dose swallowed immediately after this blood sample was drawn.

† Finger blood taken at the times noted, venous blood 5 minutes later in each case.

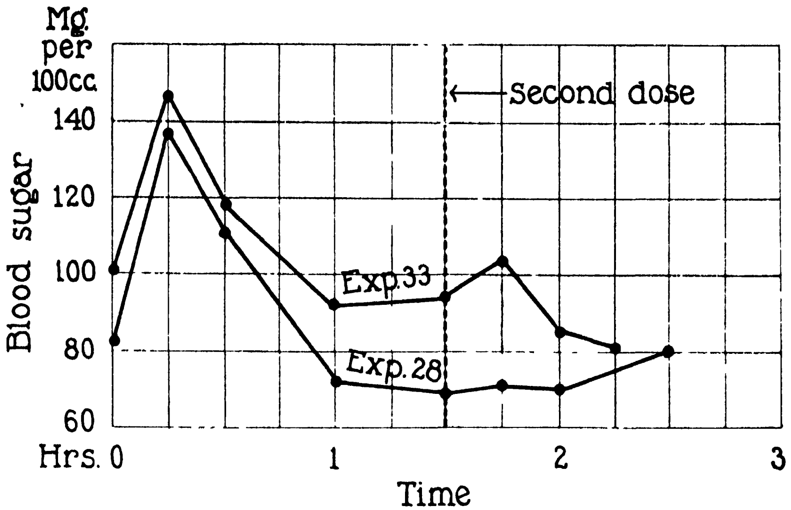

CHART 3. Showing that a second dose of glucose may have no effect on the venous blood sugar. (See Table II.)

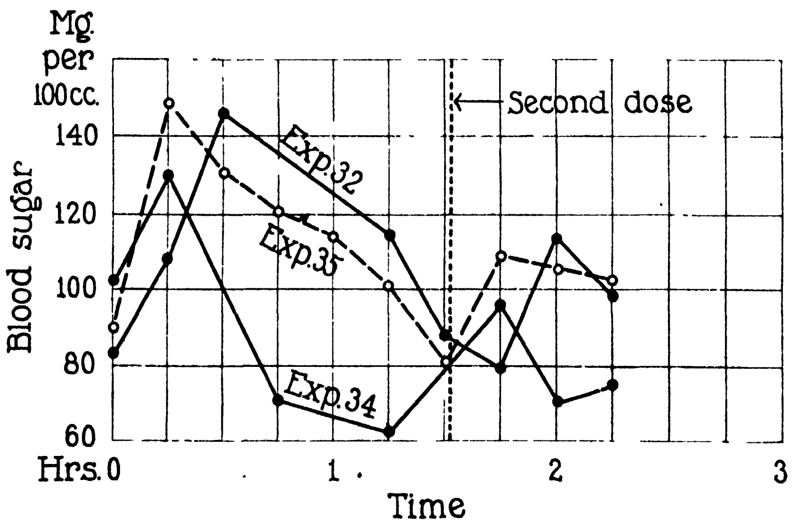

CHART 4. Showing that a second dose of glucose may cause a slight rise of venous blood sugar. (See Table II.)

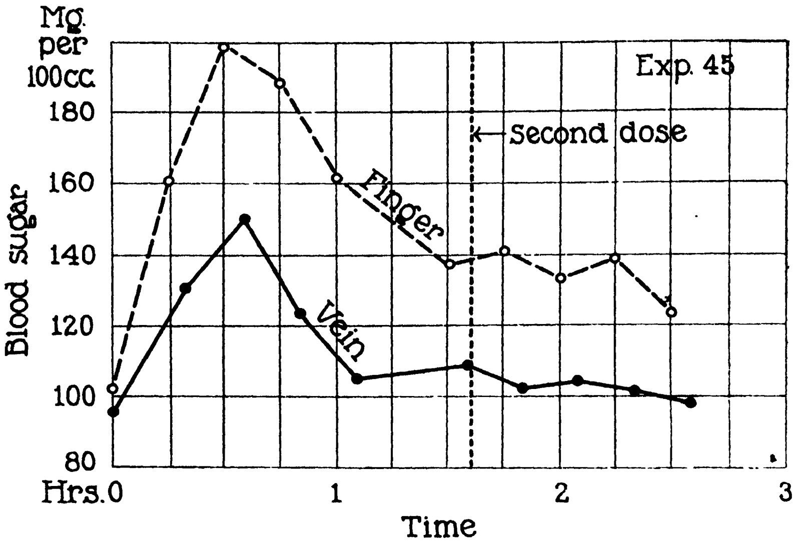

CHART 5. Showing that a second dose of glucose may have no effect on the sugar content of venous or finger blood. (See Table III.)

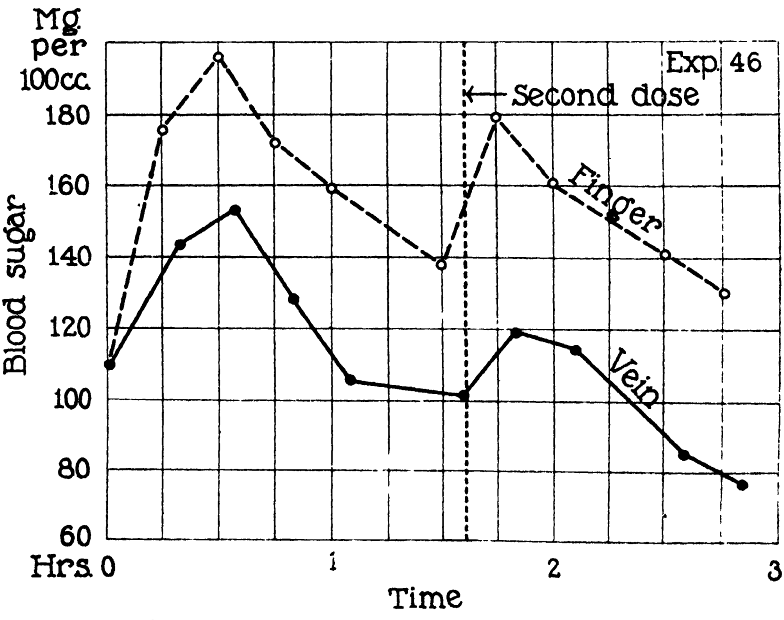

CHART 6. Showing that a second dose of glucose may cause a marked rise of sugar of finger blood with only slight effect on the venous blood sugar. (See Table III.)

DISCUSSION.

We believe that the data presented here and in the preceding paper lend support to the hypothesis of McLean and de Wesselow, the gist of which is that the existence of an alimentary hyperglycemia in the normal awakens and stimulates the glycogen-forming mechanism to such activity that not only is the rising hyperglycemia checked but that the blood sugar concentration is rapidly brought down to normal or below at a time when it is probable that glucose is still being absorbed from the alimentary tract. A somewhat similar idea has recently been expressed by Staub (6).

Standing opposed to such a view is the recent paper of Folin and Berglund (7). As a result of their blood sugar studies they conclude:

1. That it is superfluous to invoke a stimulated glycogen synthesis in explaining the nature of the curves obtained after the ingestion of glucose, but that the chief factor in preventing excessive hyperglycemia after the ingestion of sugars is simply absorption of the sugars into the general tissues, and:

2. That the frequently observed periods of hypoglycemia are not due to an overstimulated glycogenic function but occur for the reason that, the tissues in general being well loaded with readily utilizable food, there is no need for sugar transportation in the blood stream.

In reaching the first mentioned conclusion they were apparently greatly influenced by their blood sugar findings after taking galactose. They argued that if glycogen synthesis is important in checking the alimentary hyperglycemias, then galactose which is a poor glycogen former should give rise to excessively high blood sugars. In the one galactose experiment which they report, no change of blood sugar concentration was found. Such a result with galactose is, as we have pointed out before (8), entirely contrary to our findings and to the data in the literature. As shown in Table I and in the preceding paper we find excessive hyperglycemia after taking galactose. Folin and Berglund apparently continued their blood examinations for only 1 hour after taking the galactose. The urine analyses were, however, continued for a longer time and show a maximum glycuresis during the third

hour after ingestion of sugar, which coincide with the time when we find maximum hyperglycemia.

In arguing for their belief that the hypoglycemic periods represent a cessation of sugar transportation simply because the tissues are well stocked with readily utilizable food, they point to hypoglycemic levels found after the ingestion of fat and of protein. For example: 1 hour after taking 200 gm. of olive oil the blood sugar fell from 97 to 70 mg. per 100 cc. To accept this as indicating that at this time the tissues were loaded with absorbed fat, would necessitate changing some of the generally accepted views regarding fat absorption. (Bloor (9) found no increase of blood fat until 2 hours or more after feeding olive oil.) The fall of blood sugar observed by Folin and Berglund after taking gelatin was too small to have much significance (less than 10 mg.). Furthermore, Jacobsen (1) found no change in the concentration of blood sugar after feeding either fat or protein, and our own results (unpublished) agree with those of Jacobsen.

From another point of view it seems necessary to postulate a stimulation of the glycogenic function. It is not yet possible to account for the glucose$\rightleftarrows$glycogen reaction solely in terms of the concentration law. For example, consider Experiment 49 (Table I of the preceding paper). In the preliminary blood samples taken in the fasting condition, the finger blood (arterial) contained 102 mg. of glucose per 100 cc., the venous sample 101. Evidently no appreciable glycogen deposition was occurring in the muscles. 2 hours after taking the glucose we find practically the same concentration of sugar in the finger blood (106 mg.), but only 61 mg. per 100 cc. in the venous blood, which indicates a rapid glycogen synthesis. We have many cases like this in our data which indicate that some factor other than the concentration of glucose controls the rate of glycogen formation. The recent work of Banting, Best, Collip, Macleod, and Noble (10) gives us a more definite idea as to what this factor may be. They found that injection of their pancreas extracts into normal rabbits produced a remarkable fall of the blood sugar, presumably chiefly by the formation of glycogen. It is easy to assume that the ingestion of glucose or the hyperglycemia that ensues stimulates the pancreas to put out its internal secretion which then operates in some way to accelerate both glycogen synthesis and oxidation

of sugar. Such a conception would be wholly consistent with other facts of physiology. We know that the functioning of the various digestive glands is regulated according to the need for their respective products.

Further evidence which may indicate a stimulating action of glucose on the internal function of the pancreas is to be found in the very welcome paper of Bornstein and Holm (11). They found after taking 100 gm. of glucose that, whereas the blood sugar began to rise immediately, the rise in respiratory quotient exhibited a definite latent period of $\frac{1}{2}$ to 1 hour. That is, rapid increase in sugar oxidation begins at about the same time as the break in the blood sugar curve which we believe to be due to glycogen synthesis. After it has once begun, the increased rate of sugar oxidation persists for several hours, during which time the blood sugar may have fallen to normal or low levels. There is no parallelism between blood sugar concentration and the rate of sugar oxidation. On the contrary, the relation seems to be the inverse. After ingestion of 100 gm. of fructose they found the increased sugar oxidation to begin immediately with little or no rise in blood sugar.

It seems possible that the substance which is oxidized and from which glycogen is formed is not ordinary α or β glucose but some substance which is formed from ordinary glucose under the influence of the pancreatic hormone, and which is more readily formed from fructose, perhaps without the aid of the hormone.

SUMMARY AND CONCLUSIONS.

Curves of blood sugar concentration after the ingestion of glucose and of galactose are contrasted. After the ingestion of galactose the curve rises continuously for nearly 3 hours and reaches much higher levels than the glucose curve which, as is well known, rises sharply for only a short time (usually less than $\frac{1}{2}$ hour) and then rapidly falls to normal or below. It is concluded that the chief factor in preventing excessive hyperglycemia after the ingestion of glucose is glycogen formation and that the mechanism concerned in glycogen synthesis is stimulated to greater activity by the hyperglycemia which ensues after the ingestion of glucose.

Such a conclusion is essentially the same as the one previously

expressed by McLean and de Wesselow. It appears to be further strengthened by our experiments with repeated doses of glucose wherein it was found that a second dose of glucose taken soon after the hyperglycemia had subsided had little or no effect on the concentration of sugar in the blood. This fact we interpret as meaning that the first dose of glucose stimulates the glycogenic mechanism to such activity that the organism is then able to deal with any amount of glucose without becoming hyperglycemic.

Evidence is adduced to support the theory that the frequently observed periods of hypoglycemia following the ingestion of glucose are due to an "overactivity" of the glycogen-forming mechanism.

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INVESTIGATIONS ON THE IMMEDIATE EFFECT OF HEAVY EXERCISE (STAIR-RUNNING) ON SOME PHASES OF CIRCULATION AND RESPIRATION IN NORMAL INDIVIDUALS.

I. OXYGEN AND CARBON DIOXIDE CONTENT OF BLOOD DRAWN FROM THE CUBITAL VEIN BEFORE AND AFTER EXERCISE.

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This publication reports the first part of a study on the immediate effects of heavy exercise on different phases of the circulation and respiration in man. It deals with the oxygen and carbon dioxide contents of venous blood drawn from the arm during rest and about half a minute after heavy leg exercise. In subsequent papers we shall give the results of additional investigations carried out in order to enable us to analyze the experimental findings given in this publication.

Technique.

Into an oxalated glass tube, blood was drawn from a cubital vein during a period of from 15 to 40 seconds. It was collected under oil and analyzed, within 2 hours, for oxygen and carbon dioxide content in Van Slyke's apparatus. The total oxygen capacity was determined on blood saturated within the apparatus, as previously described (1).

Conditions.

After at least 10 minutes rest on a bed in a room kept at ordinary temperature, the first sample was drawn. Immediately after this, the subject ran very rapidly five times up and down a flight

TABLE I.

Oxygen and Carbon Dioxide Content of Venous Blood Drawn from the Arm after 10 Minutes Rest and Immediately after Heavy Exercise (Running Up and Down a Flight of Stairs Five Times). The Arms Were Kept Quiet during the Exercise.

Subject.			Experiment No. in protocol	Total oxygen-combining power of blood.	Oxygen content of venous blood drawn from arm		Oxygen unsaturation of venous blood drawn from arm.		Difference between oxygen content of venous blood drawn from arm during rest and after exercise	Carbon dioxide content of venous blood drawn from arm		Respiration rate.		Pulse rate.	
No.	Name.	Age.			During rest.	Immediately after exercise.	During rest	Immediately after exercise.		During rest.	Immediately after exercise.	During rest.	Immediately after exercise.	During rest.	Immediately after exercise.
		yrs.		*vol per cent*	*vol per cent*	*vol. per cent*	*vol. per cent*	*vol per cent*	*vol per cent*	*vol. per cent*	*vol per cent*				
1	O.	42	A. 1	17.74	13.83	9.60	3.91	8.14	4.23	44.41	42.83	15		84	150
2	E. M.	25	A. 2	20.78	17.00	6.98	3.78	13.80	10 02	41.84	41.84	18	40	90	120
			A. 5	20.55	17.18		3.37								
			A. 7	22.94	17.27	7.70	5.67	15.24	9.57	53 05	50.48	15	24	74	140
			A.10	19.59	15.00	5.76	4.59	13.83	9 24	55.27	55.60	14	24	80	120
			A.11	20.55	16.73	9.45	3 82	11.10	7.28	48.56	51.78	14	24	80	120
			A.23	19.82	16 75	5 98	3 07	13.84	10.77	50.68	57.14	13	32	76	120
			A.53	22.93		10.17		12.76			58.16		32		124
			A.54	22.48		6.14		16.34			58.98		36		124
			A.55	21.69		6.73		14.96			54.42		30		124
3	K. B.	32	A. 3	19.79	14 56	10.85	5.23	8.94	3.71	53.28	42.75	15	30	76	120
			A.16	19.76	11.10	6.31	8.66	13.45	4.79	57.03		18	30	78	120
4	C. M.	28	A. 4	20.08	17.86	8.40	2.22	11.68	9.46	50.30	53.72	15	26	68	150
			A. 6	19.70	14.83	6.62	4.87	13.08	8.21			15	24	62	115
5	O. M.	26	A.15	21.90	15.40	10.61	6.50	11.29	4.79	41.14	44.21	12	28	90	120
6	K. K.	27	A.17	22.23	17.78	12.56	4.45	9.67	5.22	49.91	52.64	18	26	64	92

7	E. J.	26	A.18	23.48	20.86	14.87	2.62	8.61	5.99	49.35	48.16	18	42	88	112
8	S.	28	A.19	20.08	13.16	6.31	6.92	13.77	6.85	44.10	47.63				132
9	A. J.	27	A.22	23.30	12.96	7.77	10.34	15.53	5.19	50.72	44.55		36		120
10	E. B.	30	A.24	21.38	18.80	7.46	2.58	13.92	11.34	49.54	45.18	15	28	72	144
11	H. S.	23	A.25	20.02	15.16	5.37	4.86	14.65	9.79	57.61	48.20	14	28	60	148
			A.26	19.51		4.01		15.50			47.71	14	32	70	140
12	O.	26	A.27	18.98	15.15	5.06	3.83	13.92	10.09	48.11	43.64	18	30	84	150
13	H. K.	26	A.28	21.47	15.63	6.95	5.84	14.52	8.68	49.72	44.45	11	22	66	120
14	E. H.	26	A.29	19.64	18.78	8.54	0.86	11.10	10.24	43.69	46.09				
15	P. K.	23	A.30	20.88	10.55		10.33			50.71		17	34	64	98
			A.32	19.89	9.47	5.87	10.42	14.02	3.60	50.01	49.49	16	40	60	120
16	O. H.	26	A.31	20.41	12.39	4.37	8.02	16.04	8.02	47.05	45.13	18	32	84	120
17	C. L.	37	A.12	21.13	18.26	18.10	2.87	3.03	0.16	47.22	45.16	16	48	80	130
			A.13	20.56	15.63		4.93			46.90					
			A.14	22.50	20.93	20.83	1.57	1.67	0.10		34.20	15	40	76	132
			A.20	19.71	16.51	16.96	3.20	2.75	−0.45	51.39	43.30	16	40	74	120
			A.21	21.87	18.64	19.19	3.23	2.68	−0.55	52 15	36.76	17		84	
			A.60	19.46		16.30		3.16			44.73				
			A.61	20.00		18.42		1.58			37.12				

of stairs. The arms were kept quiet, and care was taken that the arm muscles were not used. The exercise lasted from 60 to 90 seconds. From the staircase, the subject ran very fast to the bed, a distance of about 30 meters; and the second sample was drawn immediately. The drawing of the blood, therefore, was finished well within 1 minute after the exercise had ceased. During the bleeding, the pulse and respiration rates were taken.

Results.

The results are given in Table I. Thirty-five experiments were performed on seventeen normal subjects.

Oxygen of the Venous Blood.—During rest, the oxygen content of the venous blood varied over the usual wide range. The

TABLE II

Highest and Lowest Values of Oxygen Content and Oxygen Unsaturation of Venous Blood Drawn from Cubital Vein during Rest and after Exercise (Subject 17 Is Not Included).

Condition	Oxygen content of venous blood drawn from arm		Oxygen unsaturation of venous blood drawn from arm	
	Maximum	Minimum	Maximum	Minimum
	vol per cent	*vol per cent*	*vol per cent*	*vol per cent*
Rest	20.86	9.47	10.42	0.86
After exercise	14.87	4.01	16.34	8.14

highest value found was 20.93 volumes per cent (Subject 17), the lowest 9.47 (Subject 15). Similar large variations were found in the oxygen unsaturation, the lowest value being 0.86 volume per cent (Subject 14), the highest 10.42 volumes per cent (Subject 15). After exercise, the oxygen content of the venous blood was markedly decreased in sixteen of the seventeen individuals (Table II). In Subject 17 practically the same values were found during rest and after exercise. Repeated experiments on this subject gave consistent results. There was, therefore, a marked difference between the reaction to exercise of this individual and the reactions of the other sixteen. Even if this may be only a quantitative difference based on an arbitrarily chosen degree of reaction, we think that it is worth emphasizing, and shall discuss it in more detail in subsequent papers.

If only the first sixteen subjects are included, the maximum and minimum values for the oxygen content of the venous blood after exercise were 14.87 (Subject 7) and 4.01 (Subject 11) volumes per cent, respectively. The oxygen unsaturation varied between 8.14 (Subject 1) and 16.34 (Subject 2) volumes per cent.

A series of experiments were performed on Subject 2. The results were equally consistent with those on Subject 17. These two individuals, therefore, may indicate the two different types (or degrees) of reaction to heavy exercise found in our experiments. Table II gives the highest and lowest values for oxygen content and oxygen unsaturation in twenty-eight experiments on the first sixteen subjects.

Carbon Dioxide of the Venous Blood.—Only small variations were found. After exercise, the carbon dioxide content of the venous blood in some cases decreased, in others it increased. What these variations mean, and whether they were caused by exercise or by unknown and uncontrolled causes, we do not know. No further attention will be paid in this paper to the carbon dioxide content of the blood. The pulse and respiration rates need no discussion; they are included as indicating the effect of the exercise.

DISCUSSION.

The decrease in oxygen content of the venous blood present in sixteen out of seventeen cases, is outside the limits of change variations, and, therefore, is caused by variations in the experimental condition; *i.e.*, caused by the exercise performed.

We are as yet unable to explain the mechanism. That such a decrease in the oxygen content of the venous blood from resting regions can be induced by exercise, has not been shown before. No direct information can, therefore, be obtained from the literature. Two different factors suggest themselves as explanations of the fall in oxygen content of the venous blood drawn from the arm after exercise. One is a decrease in oxygen content of the arterial blood (A); another is an increased deoxygenation of the normally saturated blood, during its passage through the tissue capillaries, of the region drained by the vein from which the blood is drawn (B).

A decrease in the oxygen content of the arterial blood may be caused by a number of factors: first, by an increased acidity of the blood, caused by metabolites (lactic acid) (2, 3, 4). This would change the oxyhemoglobin dissociation curve, making it run a flatter course, so that at the same tension the blood would take up a smaller amount of oxygen. That the (reduced) pH of the blood decreased during heavy exercise, has been shown (5, 6, 7). But whether during exercise there is any relation between the reduced and the true pH is open to question. Second, the increased rate of blood flow through the lungs may decrease the degree of oxygenation of the blood (8); third, the respiratory mechanism may prove insufficient for keeping the proper oxygen tension in the alveoli. That the diffusion through the normal lung epithelium should be insufficient for the supply of oxygen during heavy exercise, can probably be excluded (9, 10).

An increased deoxygenation of the blood in the tissue capillaries of the region drained by the vein from which the blood is drawn, could be brought about, first, by an increased metabolism in the tissues drained, and second, by a decreased blood flow, which naturally could be only local. A decreased local blood flow would necessarily indicate a considerable vasoconstriction in the arm or vasodilation at other places. Such an explanation of the observed changes in the oxygen content would be in conformity with earlier determinations of the local blood flow in a resting arm during exercise with the other arm (11, 12). The difference found in the oxygen of the venous blood in different individuals after exercise would then indicate a difference in the degree to which the organism used its vasomotor mechanism during work. A further analysis of the different factors will be given, together with supplementary experiments which will be published later.

Attention should be called to the fact that, in spite of the very large oxygen unsaturation of the cubital blood after exercise, no general or local cyanosis was observed. The interpretation of this observation will be discussed in a later paper of the series. It has already been briefly mentioned elsewhere (13).

SUMMARY.

1. The oxygen and carbon dioxide contents of blood from a cubital vein have been determined in a series of normal individuals

at rest and within 1 minute after heavy exercise. The exercise consisted of running up and down a flight of stairs five times. The muscles of the arms were not used.

In sixteen subjects the oxygen of the venous blood showed a decrease far outside the variations found during rest. In one subject, the figures for the oxygen content during rest and after exercise showed no difference. The carbon dioxide content showed only small variations. In some instances a decrease, in others a small increase was found.

2. Different possible explanations of these observations are discussed briefly as an introduction to additional investigations.

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CREATININE AND CREATINE IN MUSCLE EXTRACTS.

IV. CONCERNING THE FORMATION OF CREATINE FROM METHYL GUANIDINE IN MUSCLE.

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Burns[1] and Baumann and Hines[2] could obtain no evidence validating the hypothesis that methyl guanidine can be considered a precursor of creatine.

In order to attack the problem from a slightly different angle I have incubated extracts of muscle tissue prepared as already described[3] with methyl guanidine and analyzed the extracts for their content of total creatine. Extracts buffered with phosphate mixtures to acid and alkaline reactions, with and without the addition of sodium acetate, parathyroid tissue, and extracts of liver tissue, were studied. The tissues and extracts were obtained from albino rats of both sexes, 80 to 150 days old, immediately after killing by ether.

The results were negative in all cases. No changes in the total creatinine content of the incubated extracts were obtained. The hypothesis that methyl guanidine is a precursor of creatine through intermediation of muscle activity is unsupported by these *in vitro* experiments. In view of this fact I am inclined towards the belief of Biedl[4] that methyl guanidine (supposedly found in the muscles and urine of parathyroidectomized animals) and creatine (found in the muscle tissue of normal animals) are derived from a common precursor as yet unidentified.

[1] Burns, D., *Biochem. J.*, 1916, x, 263.

[2] Baumann, L., and Hines, H. M., *J. Biol. Chem.*, 1918, xxxv, 75.

[3] Hammett, F. S., *J. Biol. Chem.*, 1921, xlviii, 133.

[4] Biedl, A., Innere Sekretion, Berlin and Vienna, 4th edition, 1922.

EXPOSURE TO LIGHT AS A SOURCE OF ERROR IN ESTIMATING URIC ACID BY THE FOLIN AND WU METHOD.

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(Received for publication, December 4, 1922.)

Attempts to secure by the Folin and Wu method quantitative recoveries of uric acid added to beef blood met at first in this laboratory with rather variable results. Although the values given by analysis were in the main satisfactory they were often too low, the losses in instances amounting to 50 per cent. A chance observation that exacting care in the measuring of reagents and prolonged stirring was associated with low results suggested that delay in the analysis might be a source of error. In view of the known properties of silver salts it was considered most likely that the uric acid was oxidized when the silver precipitate containing it was exposed to light. A study of the effect of exposure to light on the accuracy of the method was therefore undertaken.

Using a solution of uric acid made to approximate a blood filtrate and conducting the analysis in a dark room, a recovery of 97 per cent was secured. An immediate repetition of the analysis in a well lighted laboratory gave a recovery of only 79 per cent. In this case an attempt was made to imitate conditions under which the method might often be used. In other instances, excessive losses were obtained by prolonging the exposure. The losses of uric acid so obtained were not due to an incomplete precipitation or to an incomplete liberation from the precipitate for it was repeatedly found that even with the greatest losses the solution decanted after the first centrifuging was negative qualitatively for uric acid, and the silver residue after the second centri-

* The author acknowledges his indebtedness to Prof. B. B. Turner for valuable instruction and advice.

fuging when dissolved in cyanide also gave a negative qualitative test.

In order to make comparisons between effects secured when the intensity of light and the duration of exposure were both variables an actinometer,[1] designed for use in amateur photography, was used to measure the intensity of the light and the exposures were calculated in arbitrary "actinic units;" *i.e.*, the duration of the exposure in seconds was divided by the time in seconds required for the sensitive paper in the actinometer to match the darker of the two tints on the dial. A curve, plotted with actinic units of exposure as abscissæ and loss in percentage as ordinates, shows that with slight exposures the loss is nearly proportional to the exposure, but that as the amount of uric acid in the solution is reduced the action proceeds more slowly. Time has been lacking for an adequate physicochemical study of the rate at which the oxidation proceeds as this seems to be affected by a number of factors other than the intensity of the light and the duration of the exposure; *e.g.*, frequency of stirring and possibly temperature.

By using 5 per cent silver lactate in 2.5 per cent lactic acid and 5 per cent silver lactate in 10 per cent lactic acid as well as the usual 5 per cent silver lactate in 5 per cent lactic acid in analyses conducted simultaneously both in the dark room and with a prolonged exposure to light it was found that an increase in the hydrogen ion concentration increases the loss from exposure. This is a point in favor of the partly neutralized lactic acid silver lactate reagent recently described by Folin.[2] That the use of the latter reagent does not obviate the danger of incurring losses from the action of light has been demonstrated in Experiment 7.

The effect of an exposure made after treating the precipitate containing uric acid with 10 per cent sodium chloride in 0.36 per cent hydrochloric acid is much less than the effect of an equivalent exposure made before this treatment. It is similar to the effect found to occur on exposing to light a blood filtrate mixed with a small amount of washed silver chloride (Experiment 4). That losses from equivalent exposures are lower in this case is probably due to the fact that the uric acid is in solution; the action probably taking place at the surfaces of the particles of silver precipitate

[1] A Wynne's "Infallible" Exposure Meter was used.

[2] Folin, O., *J. Biol. Chem.*, 1922, liv, 153.

and proceeding rapidly while the uric acid is adsorbed at those surfaces, but much more slowly when it must be brought in contact with them by diffusion.

Results obtained by analyzing both with and without exposure to light the tungstic acid filtrate from beef blood to which uric acid had been added and from human blood have been in accord with those obtained by using uric acid solutions.

From these observations it would seem that the estimation of uric acid in blood by the Folin and Wu method is likely to give low results if conducted at ordinary speed in well lighted laboratories. On a dull winter day in a laboratory with a northern exposure the light may be so poor in actinic quality that if the exposure is limited to 2 or 3 minutes the losses may not be appreciable. But during the summer months and in well lighted laboratories the only safe method is to keep the centrifuge tubes with their contained silver precipitates constantly within trunnion-cups or in some other manner shielded from light until the redissolved uric acid has been decanted from them. The methods described by Benedict[3] and by Folin[2] for the estimation of uric acid in the blood filtrates directly are, of course, not subject to the same source of error but if the separation of the uric acid from other substances is retained, as Folin suggests, for a control method, the same precautions must be taken as in the regular Folin and Wu procedure.

EXPERIMENTAL.

In the experimental analyses reported below a freshly prepared 0.0005 per cent solution of uric acid in a 0.05 per cent solution of sodium chloride has, unless otherwise stated, been used instead of a tungstic acid blood filtrate. This was done in order to measure with absolute certainty the losses which occurred. The procedure described by Folin and Wu was followed except that in most instances 5, 10, 15, and 20 cc. portions of a freshly prepared solution of uric acid placed in 50-cc. volumetric flasks were used as standards instead of 1 and 2 cc. portions of the standard they describe. In this way the use of sodium sulfite was avoided, with a resulting increase of color development that was of advantage, particularly when considerable losses were encountered.

[3] Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.

Experiment 1.—An analysis conducted in a dark room by a safe light, such as is used in photography for manipulating "developing out" paper, was immediately repeated in a well lighted laboratory. An attempt was made to imitate conditions under which the method might often be used, the analysis being conducted fairly rapidly but without any precautions to prevent exposure of the silver precipitate to light. The actinometer time at the table at which the analysis was conducted was about 30 seconds. Approximately 10 minutes elapsed between the addition of the silver lactate in lactic acid reagent and the transference of the liberated uric acid from the centrifuge tubes to the 25 cc. volumetric flask. The results were as follows:

Analysis.	Error.
	per cent
In dark room	3
" light laboratory	21

Experiment 2.—In a series of analyses the silver precipitates containing uric acid were exposed to light for varying intervals. The results are shown in Table I. In each instance the silver residues after treatment with 10 per cent sodium chloride in 0.36 per cent hydrochloric acid when dissolved in cyanide gave a negative qualitative test for uric acid as did also the solution decanted after the first centrifuging. The actinometer time was determined in each instance, but unfortunately the stirring was not uniform and the temperature was not taken. As exposure was found to have much more effect immediately after the addition of the silver lactate lactic acid reagent than after the liberation of the uric acid; most of the exposures were made at that stage.

Experiment 3.—In order to test the influence of hydrogen ion concentration on the light action solutions of 5 per cent silver lactate in 2.5 per cent lactic acid and 5 per cent silver lactate in 10 per cent lactic acid were prepared in addition to the usual 5 per cent silver lactate in 5 per cent lactic acid. Determinations were made with these reagents, first in a well darkened laboratory, then with 15 minutes exposure to light having an actinometer time of about 13 seconds. The results are given in Table II.

The 5 per cent silver lactate in 2.5 per cent lactic acid gave the minimum losses and the 5 per cent silver lactate in 10 per cent lactic acid the greatest. These results indicate that an increase in the hydrogen ion concentration increases the loss from exposure to light. The results in the darkened laboratory indicate that it also tends to make the precipitation of uric acid less complete.

Experiment 4.—In each of two centrifuge tubes were placed 2 cc. of 5 per cent silver lactate in 5 per cent lactic acid and 1 cc. of 10 per cent sodium chloride in 0.36 per cent hydrochloric acid. The resulting precipitate of silver chloride was washed several times by stirring up with distilled

water, centrifuging, and decanting. To the washed silver chloride in each tube were then added 10 cc. of tungstic acid blood filtrate and the mixture was thoroughly stirred. The tubes were then left exposed 20 minutes to light, having an actinometer time of about 20 seconds. The blood filtrate was then separated from the silver chloride by centrifuging, transferred to other tubes, and analyzed by the regular Folin and Wu procedure. The

TABLE I.

Conditions relating to exposure.					Loss.
Stage of analysis.	Actinometer time.	Duration.	Actinic units.	Stirring.	
	sec.	*sec*			*per cent*
After addition of silver lactate in lactic acid reagent.	7	300	43	Every 5 min.	77
	7	1,800	257	" 5 "	92
	35	70	2	" 5 "	40
	32	140	44	" 5 "	60
	23	180	78	" 5 "	64
	100	100	1	Constant.	16
	80	160	2	"	38
	135	410	3	"	30
	150	750	5	"	48
	25	200	8	"	62
	40	480	12	"	82
	40	900	22	"	78
After addition of 10 per cent sodium chloride in 0.36 per cent hydrochloric acid and water.	7	900	129	Every 5 min.	38

TABLE II

Reagent used.	Loss.	
	Without exposure.	With exposure
	per cent	*per cent*
5 per cent silver lactate in 2.5 per cent lactic acid ...	3 8	75
5 " " " " " 5 per cent lactic acid......	5.4	83
5 " " " " " 10 per cent lactic acid	6 0	88

value found was 3.44 mg. per 100 cc. of blood. The silver chloride was treated with 10 per cent sodium chloride in 0.36 per cent hydrochloric acid, but no uric acid was liberated from it. The same variation was tried in a dark room. The value obtained was 4.55 mg. per 100 cc. of blood. An analysis in a dark room by the regular Folin and Wu technique gave 5.00 mg. per 100 cc. of blood.

Experiment 5.—To a 10 cc. portion of beef blood in a flask were added 50 cc. of a 0.001 per cent solution of uric acid and 20 cc. of water. A tungstic acid filtrate was then prepared in the usual way and designated as Filtrate 1. To another 10 cc. portion of the same blood in another flask were added 30 cc. of the 0.001 per cent solution of uric acid and 40 cc. of water. A tungstic acid filtrate was then prepared and designated as Filtrate 2. Designating by x the number of milligrams of uric acid in 100 cc. of the blood, then Filtrate 1 should yield by analysis $x + 5$ mg. per 1,000 cc. (equivalent to 100 cc. of blood) and Filtrate 2 should yield $x + 3$ mg. per 1,000 cc., and the difference between the values obtained from the two filtrates should be just 2 mg. per 1,000 cc. The results obtained by analyzing both filtrates in a dark room and Filtrate 1 under varying conditions of exposure to light are shown in Table III.

TABLE III

	Per 1,000 cc.
	mg.
Filtrate 1.	
(a) In dark room rapidly	5.69
	5.82
(b) In dark room. Exposed to safe light for 30 minutes after addition of silver lactate lactic acid reagent	5.61
(c) In light laboratory. Exposed 210 seconds (actinometer time 20 seconds) after addition of silver lactate in lactic acid reagent	3.74
(d) In light laboratory. Protected by keeping centrifuge tubes in trunnion-cups and covered with tin-foil, except while stirring	5.54
Filtrate 2.	
(a) In dark room rapidly	3.65
	3.68

The difference between the average values obtained for the two filtrates in the dark room was 2.09 mg. per 1,000 cc. which probably represents a small positive error present in the 5.82 figure, the value for x being probably about 0.70 to 0.75 mg. A little exposure to light produced a very serious error (about 40 per cent). A satisfactory result could, however, be obtained in a light laboratory if care was used to shield the centrifuge tubes from light with trunnion-cups and tin-foil until the uric acid could be removed from contact with the silver precipitate.

Experiment 6.—The tungstic acid filtrate from a specimen of human blood was analyzed, first in a well darkened laboratory, and again with an exposure of 3 minutes to light having an actinometer time of about 25 seconds. The values obtained in terms of milligrams of uric acid per 100 cc. of blood were:

(1) Without exposure.. 2.52
(2) With exposure.. 1.91

The result in this case indicates that conclusions drawn from the results obtained with uric acid solutions and with beef blood are applicable to the analysis of human blood.

Experiment 7.—To a 10 cc. portion of beef blood in a flask were added 50 cc. of a 0.001 per cent solution of uric acid and 20 cc. of water. A tungstic acid filtrate was then prepared in the usual way. Two analyses were then done upon the filtrate by the method recently described by Folin[3] using the partly neutralized lactic acid silver lactate solution to separate the uric acid from other substances. In one analysis the centrifuge tube containing the silver precipitates was exposed 5 minutes to light having an actinometer time of about 25 seconds; in the other, care was taken to shield the tube from light. The results expressed as milligrams per 1,000 cc. of filtrate were:

(1) Without exposure.. 5.36
(2) With exposure 2.00

CONCLUSIONS.

1. Exposure to light may be a very serious source of error in estimating uric acid in blood by the Folin and Wu method.
2. The effect of light is most marked at the time the uric acid is present in the silver precipitate.
3. The loss is approximately proportional to the exposure until the amount of uric acid is appreciably reduced, when the action proceeds more slowly.
4. Increasing the hydrogen ion concentration of the precipitating reagent increases the losses.
5. The new method of Folin is subject to the same source of error if the uric acid is separated from the other constituents of the blood filtrate.
6. Error from this source is simply and easily obviated by shielding the centrifuge tubes from light with trunnion-cups and tin-foil until the liberated uric acid has been decanted from them.

A NEW METHOD FOR THE SEPARATE EXTRACTION OF VACUOLE AND PROTOPLASMIC MATERIAL FROM LEAF CELLS.*

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(Received for publication, January 26, 1923.)

During the past 2 or 3 years attempts have been made in this laboratory and elsewhere to gain some knowledge as to the chemistry of the nitrogenous products contained in the leaf cell.[1] To extract these, methods have been so devised that the major part of the leaf cells have been mechanically ruptured by grinding with water, whereby all the substances contained in the vacuole, with a large part of the protoplasmic membrane, are obtained in one complex colloidal solution. By heating to about 60° or by the addition of alcohol it was found that the colloidal substances in the extract could be caused to flocculate. On analysis these "colloidal precipitates" were found to consist in large part of protein, but, as one would expect from such a complex, other substances having similar physical properties were also present, and the subsequent separation and purification of the protein was a matter of some difficulty.

It was therefore resolved to attack the problem from a different standpoint, and if possible, extract the fluid vacuole content with-

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The author wishes to express his thanks to Dr. Thomas B. Osborne for his interest in this work and also for much helpful advice and criticism.

[1] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1920, xlii, 1. Osborne, T. B., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, 1921, xlix, 63; 1922, liii, 411. Chibnall, A. C., and Schryver, S. B., *Biochem. J.*, 1921, xv, 60. Chibnall, A. C., *Biochem. J.*, 1922, xvi, 344.

out rupturing the cells, so as to leave the protoplasmic membrane intact. To do this it was decided to plasmolyze the leaf cells, and see if the vacuole content could be squeezed out by pressure without damage to the cell walls. This method is not new. It was first successfully employed in 1913 by Dixon and Atkins[2] in their investigations into the osmotic pressure of cell saps. André, a year or so previously, had called attention to the fact that the sap obtained by pressure from untreated fresh leaves did not represent in composition that of the fluid content of the vacuole, since the more easily diffusible substances were the first to pass out. Further, to obtain any large proportion of the sap great pressure was required, which caused rupturing of the cells, thereby allowing part of the protoplasm to escape. Dixon and Atkins killed the protoplasmic membrane by freezing the tissues with liquid air. After thawing they found that the sap was very easily expressed by moderate pressure. The successive fractions of juice contained similar concentrations of soluble material, as shown by the depression of the freezing point. They considered that this represents the fluid vacuole content of the cells. This method has since been repeatedly employed by Gortner and his coworkers for the examination of the osmotic pressures of cell saps.

As treatment of large quantities of leaves by this method is inconvenient, it was decided to kill the cells by means of certain organic fluids, which were known to have a toxic action on the protoplasmic membranes.

In 1909 Guignard[3] and Mirande[4] showed that the vapors of chloroform and ether caused the exudation of liquid from cells. The following year Czapek[5] produced evidence that liquids having a surface tension in air of less than 0.68 (water as unity) caused irreversible plasmolysis. Osterhout,[6] in his account of experiments with *Laminaria*, directed towards an explanation of the

[2] Dixon, H. H., and Atkins, W. R. G., *Scient. Proc. Roy. Dublin Soc.*, 1913, xiii, 422.

[3] Guignard, L., *Compt. rend. Acad.*, 1909, cxlix, 91.

[4] Mirande, M., *Compt. rend. Acad.*, 1909, cxlix, 140.

[5] Czapek, F., *Ber. bot. Ges.*, 1910, xxviii, 480; Ueber eine Methode zur direkten Bestimmung der Oberflachenspannung der Plasmahaut von Pflanzenzellen, Jena, 1911.

[6] Osterhout, W. J. V., *Science*, 1913, xxxvii, 111; *Bot. Gaz.*, 1916, lxi, 149.

action of anesthetics on the permeability of cell membranes, stated that if concentrated aqueous solutions of ether or chloroform were used, a rapid fall in the permeability, ultimately resulting in the death of the cell, was observed in 30 seconds.

As far as the author is aware, however, no one has yet called attention to the extreme rapidity with which these organic liquids, themselves, in contradistinction to their aqueous solutions, cause plasmolysis of leaf cells. Preliminary experiments showed that

TABLE I.

Action of Various Liquids on the Turgidity of Spinach Leaves.

Space = fully turgid.
− = starting to become flaccid.
\+ = flaccid, thick stems still a little turgid.
++ = whole leaf, with stems, completely flaccid.

Substance.	Time in minutes.					
	¼	½	1	2	4	10
Ether water					−	+
Ether	+	++				
Petroleum ether		−	−	−	−	−
78 per cent alcohol						
Absolute alcohol				−	−	+
Butyl alcohol	−	−	+	+	+	++
Capryl alcohol	+	++				
Benzene		−	+	+	+	+
Toluene			−	−	−	+
Xylene						
Chloroform	+	++				
Acetone			−	+	+	+
Butyl aldehyde		−	+	++		
Ethyl acetate		−	+	+	+	+
Methyl salicylate				−	−	+

if fresh turgid spinach leaves were placed in ether, they were rendered extremely flaccid in a few seconds. A similar result was obtained with soft leaves from other plants, extreme flaccidity resulting after immersion in ether for about 1 minute. In certain cases when the leaves were stiff, and had a relatively large epidermis, such as *Begonia sanguinea* the period required was very much longer, probably due to the slower penetration of the ether. Other plasmolyzing agents have been tried, but in most cases they were not so rapid. Their action is illustrated by Table I.

Further observations were made as follows: Isolated cells from freshly picked spinach leaves were teased out. Viewed under the microscope the cells were seen to be turgid, the protoplasmic layer, in which the chloroplasts could be distinctly seen, lining the inside of the cell wall. A drop or two of ether was then run between the glass slide and the cover-slip. Contraction of the protoplast away from the cell wall (to about half the original volume) was almost instantaneous. All movement ceased within 5 seconds.

The author can find only two recorded instances of plasmolyzing agents (other than cold) being used to facilitate the extraction of cell contents. Dixon and Atkins[2] tried exposing leaves to the vapors of chloroform and ether, but as the time required for complete plasmolysis was about 40 hours they preferred the more rapid action produced by cooling. Giglioli[7] exposed yeast to the vapors of chloroform for 3 to 5 hours and found that liquid slowly exudes. He was then able to filter off a fluid showing small zymase activity. In the following pages the author illustrates the separation of the vacuole content of the cells of spinach leaves by pressure, following preliminary plasmolysis.

EXPERIMENTAL DETAILS.

Experiment I.—This was a preliminary experiment designed to find out what separation between the vacuole content and protoplasm could be obtained.

About 1 kilo of spinach leaves was immersed in ether for about a minute. The mass of plasmolyzed leaves was then enveloped in filter cloth and pressed between flat iron plates in a Buchner press.

A clear brownish red liquid was obtained (Extract I), which was free from suspended matter but gave a slight Tyndall effect. The total absence of the green colloidal material, characteristic of the extracts made by the earlier grinding methods, showed that very little, if any, protoplasmic material was present.

The addition of two-fifths volume of 78 per cent alcohol to Extract I produced a white precipitate on standing, which contained only 2.4 per cent of N. It had 31.5 per cent of ash, which contained Mg and PO_4. The clear alcoholic filtrate, after removal of the alcohol, gave a small coagulum on boiling. This contained 14.5 per cent of N, indicating the presence of small quantities of protein in the original fluid.

[7] Giglioli, I., *Atti Accad. Lincei rend.*, 1911, xx, pt. 2, 349; *Arch. ital. biol.*, 1912, lviii, 437.

The residue in the press was a firm thin cake, easily separated from the filter cloth. Microscopic examination showed that the flattened cells were unruptured, and still contained their protoplasm. The individual chloroplasts, however, could no longer be distinguished, either because they had been diffused into the general protoplasmic mass by the pressure, or because green color due to the chlorophyll had been dispersed by the solvent action of the ether. The pressed cake was then allowed to imbibe water, which it did very readily, passed three times with water through the Nixtamal mill, and the resultant mixture pressed out. This process was repeated five times in all, the first two extracts being united to form Extract II and the last three united to form Extract III. Extract II was green and heavily charged with colloidal material—similar to the extracts obtained by the older grinding methods. The addition of a few drops of glacial acetic acid caused the flocculation of a colloidal precipitate, which, after washing with alcohol and ether, contained 14.2 per cent of N; 5.1 per cent of ash; or 15.0 per cent of N, ash-free. Extract III gave a similar preparation.

This experiment was sufficient to throw considerable light on the chemical constitution of the leaf cell and of the nature of the "colloidal precipitates" that had previously been isolated from them.

In the first place the vacuole fluid of the cell contains only traces of protein (1 to 2 per cent of the total leaf N). It does, however, contain quantities of organic and inorganic phosphates. These have, in the older methods of extraction, been precipitated in the colloidal precipitate, thereby reducing the proportion of N in these preparations to from 10 to 13 per cent.[8]

In the second place the colloidal precipitates obtained in the present experiment came from the protoplasm of the cell. It is composed of substances soluble in alcohol and ether, together with a complex containing very little other than protein, as shown by the high content of N (15 per cent).

Experiment 2.—In some experiments that are not recorded here it had been observed that repeated washing of the pressed residues caused the solution of part of the leaf proteins. Accordingly, in the present experiment 800 gm. of clear turgid leaves were treated with ether and pressed out. The pressed residue was allowed to imbibe about 500 cc. of water and again pressed out. This operation was repeated. The total N in each of these three extracts was then taken; they were then united and boiled

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[8] Compare Osborne, T. B., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, 1921, xlix, 63.

(without preliminary precipitation of the phosphate complex with alcohol). A small coagulum appeared, which, after washing and drying, contained 12.5 per cent of N or 1 to 2 per cent of the total leaf N. The residue from the press was then ground with water in the Nixtamal mill as before, the mixture being filtered through paper pulp instead of being pressed out. The clear solution so obtained (Extract IV) was yellow-brown in color. The addition of a small quantity of acid caused a fairly heavy flocculation, the whitish precipitate so formed being soluble in a small excess of either acid or alkali. After washing with alcohol and ether it contained 14.9 per cent of N or 15.15 per cent of N, ash-free. The above grinding with water and filtration was repeated four times. The second solution (Extract V) gave a small quantity of protein, containing 15.4 per cent of N or 15.65 per cent of N, ash-free. Without concentration the other extracts were too dilute to remove the protein by flocculation. They both gave a strong biuret reaction, and on concentration gave a coagulum too small for examination.

Table II shows the distribution of solids and nitrogen so obtained. As filter pulp had been used for filtration, it was impossible to obtain the dry weight of the ultimate residue.

This experiment brings out the following points:

1. After removal of the water-soluble substances present in the vacuole, which would appear to act as a buffer, part of the protoplasmic protein, amounting to about 14 per cent of the leaf N or 20 per cent of the total protein N, is soluble in water.

2. The water-soluble substances in the vacuole contain a little protein, about 1 to 2 per cent of the total leaf N, which has different properties than that obtained from the protoplasm.

3. For a complete extraction of the non-protein water-soluble substances some method of preventing the solution of the protoplasmic proteins must be used.

Experiment 3.—This experiment was carried out with the idea of separating all the simpler water-soluble substances of the leaves without solution of the proteins. To do this the pressed residues were allowed to imbibe 0.002 N HCl in place of distilled water, as it had been found in the previous experiments that this concentration of acid caused the flocculation of the proteins extracted by distilled water.

On account of the very complete plasmolysis of the cells by the ether the surface of the leaves, when placed in this liquid, became moist, due to excosmosis of the vacuole content. Some of this aqueous fluid drops off the surface of the leaves and collects at the bottom of the beaker in which the leaves are being plasmolyzed. In previous experiments this fluid had been neglected—in the present it was recovered and is denoted henceforth as the "exudate." The pressed residues were allowed to imbibe the dilute

acid four times, no grinding taking place. Table III shows the distribution of total solids and N obtained.

The residue from the fourth extract, after imbibing the dilute acid, stood over night. Slight solution or autolysis of protein occurred, as is

TABLE II

Showing the Distribution of Nitrogen in 800 Gm of Spinach Leaves, after Preliminary Plasmolysis with Ether and Subsequent Extraction with Distilled Water

	Total solids	Total N	Percentage of total N	Percentage of N
Vacuole content				
	gm	*gm*	*per cent*	*per cent*
Expressed before grinding				
Extract I	19 53	0 782	18 35	4 00
" II	4 79	0 236	5 54	4 93
" III	3 26	0 245	5 74	7 52
Total	27 58	1 263	29 63	4 58
Protoplasmic material				
Protein, extracted by distilled water after grinding				
Extract IV	2 46	0 366	8 59	14 9
" V	0 70	0 108	2 54	15 4
Remaining in solution after precipitation of the protein (biuret reaction positive in each extract)				
Extract IV		0 077	1 81	
" V		0 057	1 34	
" VI		0 016	0 37	
" VII		0 027	0 64	
Remaining in residue		2 347	55 08	
Total		4 261	100 00	

shown by the rise in the percentage of N in the fifth extract, and the fact that a biuret reaction was readily given by the latter

A similar separation, using butyl alcohol as a plasmolyzing agent, is given in Table IV (the leaves were from a different sample), the pressed residues being allowed to imbibe the 0 002 N HCl for about 15 minutes each time.

TABLE III

Extraction of the Vacuole Content of about 1.2 Kilos of Washed Spinach Leaves (Picked 2 to 3 Days) after Preliminary Plasmolysis with Ether, and Subsequent Extraction with 0 002 N HCl.

	Total solids		Total N		N
	gm	*per cent*	*gm*	*per cent*	*per cent*
Exudate	4 86	4 59	0 208	3 44	4 28
1st extract	27 30	25 80	1 130	18 67	4 14
2nd "	4 86	4 59	0 208	3 44	4 28
3rd "	2 58	2 44	0 119	1 97	4 61
4th "	1 23	1 16	0 058	0 96	4 72
5th "	2 53	2 39	0 138	2 28	5 45
Total extract	43 36	40 97	1 861	30 76	4 29
Residue	62 49	59 03	4 190	69 24	6 70
Total leaf	105 85	100 00	6 051	100 00	5 71

This experiment brings out the following points.

1. The exudate, with Extracts 1 and 2, contains the bulk of the vacuole content. That the method of extraction gives samples of sap which are of similar composition, presumably that of the sap *in vivo*, is shown by the similarity of the percentage of nitrogen in them. For observations on the lowering of the freezing

TABLE IV

Extraction of the Vacuole Content of 1 Kilo of Washed Spinach Leaves (Picked 2 to 3 Days) after Preliminary Plasmolysis with Butyl Alcohol, and Subsequent Extraction with 0 002 N HCl.

	Total solids		Total N		N
	gm	*per cent*	*gm*	*per cent*	*per cent*
Exudate	2 60	2 87	0 108	2 20	4 14
1st extract	23 45	25 84	0 917	18 64	3 91
2nd "	6 80	7 51	0 272	5 53	4 00
3rd "	2 89	3 19	0.096	1 95	3 32
4th "	1 57	1 74	0 058	1 18	3 69
5th "	0 83	0 92	0 034	0 69	3 32
Total vacuole content	38 14	42 07	1 485	30 19	3 90
Residue	52 50	57 93	3 434	69 81	6 54
Total leaf	90 64	100 00	4 919	100 00	5 43

point it is not necessary to extract the whole vacuole content; part will suffice, provided that it can be accepted as representative. Now the first extract from the press contains more than half the vacuole content in the undiluted state (leaving for a moment the fact that ether is appreciably soluble in water)—more than sufficient for any freezing point determination. The plasmolytic method then, if an agent insoluble in water, such as benzene be employed, is a very rapid method that may possibly be used to replace that of Dixon and Atkins, which requires troublesome freezing.

2. The separations given in Tables III and IV contain:

(*a*) An extract containing *all* the vacuole content, with possibly some constituents of the protoplasm, which, at the time of death, have either passed into the vacuole, or have subsequently been washed out of the protoplasm by the dilute acid; and (*b*) a residue consisting of cell wall material, together with the cell protoplasm, from which some of the constituents may have been removed, as per (*a*) above.

In the present paper the separation of the constituents of the leaf cell by a new method is described, which appears to offer a better opportunity for the chemical examination of cell protoplasm, as well as of the vacuole content, in bulk, than has hitherto been available owing to the experimental difficulties involved. If suitable plasmolyzing agents are employed the method may possibly be of use in the extraction of zymase from yeast and the cell content of animal tissues.

SUMMARY.

A new method for extracting the vacuole content of cells is described, and its application to the separation of the cell content of spinach leaves illustrated.

This consists of plasmolyzing the cells by means of certain organic agents, such as ether, butyl alcohol, etc., and then pressing out the major part of the vacuole content in the Buchner press. The remainder of the vacuole content can be washed out by repeatedly allowing the pressed residues to imbibe very dilute acid (0.002 N) and subsequently pressing.

This operation does not rupture the leaf cells, and the protoplasm, from which some of the water-soluble constituents may have been washed out, is retained within them.

After removal of the vacuole content, the protoplasmic material remaining in the residue can be extracted by grinding with water. It passes into colloidal solution and can subsequently be flocculated by the addition of acid. It is composed of substances soluble in alcohol and ether, together with a complex the greater part of which is protein.

If distilled water is used instead of dilute acid for washing the pressed residues, it is found that part of the protoplasmic protein passes into solution. This can be flocculated by the addition of acid, and the precipitate which contains 14.9 per cent of N (ash-free 15.15 per cent of N) is soluble in small excess of either acid or alkali.

The new method appears to offer a better medium for the chemical examination of cell protoplasm, as well as of the vacuole content, than has hitherto been described.

THE GRAVIMETRIC DETERMINATION OF ORGANIC PHOSPHORUS.

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It has been shown that many problems concerning the chemical as well as the physiological decomposition of nucleic acid, which otherwise present no obvious point of attack, may be solved easily by following the phosphorus. Studies of this nature made over a long period of time have resulted in what we believe to be a marked improvement in the older method of phosphorus determination and have produced an accumulation of interesting data that can be conveniently included under the above title.

Some of the conclusions now stated were contained in the essay of Mary L. Mall which was submitted for the Master's degree in this University in 1922, and scattered fragments have occasionally been published, but the bulk of the material in coherent form appears here for the first time.

The conventional determination of phosphorus in organic compounds involves the following processes.

1. The organic matter must first be oxidized and by a process which insures the conversion of phosphorus into its highest oxide. This was formerly accomplished by a cumbersome dry fusion of the material which necessitated the use of silver apparatus and the subsequent removal of silver from the product; but an advance was made by Neumann[1] in 1903 when he introduced his method of "wet ashing." Though a great improvement upon the older method, Neumann's procedure requires constant attention, is unpleasant in its execution, and unless habitually done, is attended with some uncertainty.

[1] Neumann, A., *Z. physiol. Chem.*, 1902-03, xxxvii, 115.

In place of this universally employed procedure of Neumann we propose to burn the organic substance with concentrated sulfuric acid, potassium sulfate, and a trace of copper sulfate just as though a Kjeldahl nitrogen determination were to be made. This involves an operation that is familiar to everyone and requires only material and apparatus that are obtainable in every laboratory. No after treatment is necessary, it being sufficient to dilute the "Kjeldahlized" product with 8 to 10 parts of water as it is quantitatively transferred to a short necked flask suitable for precipitation with molybdic solution.

2. All gravimetric methods involve precipitation of the phosphoric acid with molybdic solution for the purpose of removing soluble bases (not ammonium compounds). The yellow, easily filterable precipitate having approximately the composition $(NH_4)_3PO_4 \cdot 12MoO_3 \cdot 2HNO_3 \cdot H_2O$ owes its advantage to its visibility, its demonstrated insolubility, and its low percentage of phosphorus (about 1 per cent), so that the propriety of its intermediate use in an analytical scheme is scarcely worth considering. However, various attempts have been made to shorten the procedure by weighing the yellow precipitate as an end-product, but they are all unsatisfactory in so far as they involve some unnatural factor for calculating the weight of phosphorus from the weight of the yellow precipitate and indeed authorities differ among themselves as to the magnitude of this factor.

3. It is customary to dissolve the yellow precipitate in ammonia and to precipitate the phosphoric acid from the solution as magnesium ammonium phosphate by the addition of magnesia mixture.

We shall show how magnesium ammonium phosphate can be precipitated in the form of large stable crystals of constant composition which can easily be dusted from a filter paper when dried in the air.

4. It is the universal custom to convert the dry magnesium ammonium phosphate into magnesium pyrophosphate by intense heating and from the weight of the latter to calculate the corresponding weight of the phosphorus. This presupposes the burning of a filter paper that has been washed free from soluble constituents with the greatest care unless one is disposed to accept the annoyance and uncertainty of a porcelain Gooch (platinum apparatus should not be used in this connection).

We propose to show that magnesium ammonium phosphate is a very stable compound represented by the formula $MgNH_4PO_4 \cdot 6H_2O$ which does not change its composition under ordinary conditions in any length of time. It is so easily obtained and preserved that it not only serves as an excellent end-product for phosphorus determination but as will be shown, may be employed in the preparation of standard solutions of any one of its constituents and can be used with great advantage in rapidly preparing exact solutions of standard acids. The above suggestion eliminates the most costly and tedious steps in the determination of phosphorus.

The advantages of the alterations suggested are obvious, their correctness remains to be proved.

In rigidly testing a method, agreement in the results of parallel determinations made with unknown or complicated mixtures will not answer: determinations should be made either with pure crystalline compounds and the results compared with the requirements of a formula or they should be made with standard solutions that have been standardized by processes and with reagents which will not afterwards be employed in the execution of the method. Moreover, a method should be tested in its various parts lest several errors of opposite sign should balance one another and not do so on some subsequent occasion. In securing the analytical data that follows an attempt was made to fulfil these conditions.

Preparation of a Standard Solution of Phosphoric Acid.

A 10 per cent solution of ammonium phosphate, faintly acidified with acetic acid, was slowly precipitated at the boiling point with neutral lead acetate. The precipitated lead phosphate was filtered off tightly with a Buchner and repeatedly washed by removal from the funnel and grinding with hot water until both acetic acid and ammonium salts had been completely removed. This procedure finally leads to a preparation in which no trace of either acetic acid or ammonium salts can be shown by the most sensitive tests. The lead phosphate was then suspended in hot water, decomposed with hydrogen sulfide, and the filtrate from lead sulfide was evaporated under diminished pressure to a small volume. The syrupy residue of phosphoric acid was then taken up in absolute alcohol, filtered from a trace of insoluble flocculent material, and after evaporation of the alcohol, the pure phosphoric acid was

diluted with water to a strength convenient for the experiments described below.

The purity of the product is easily tested. Phosphoric acid is for practical purposes a dibasic acid having two ionization constants which differ from each other enormously. The values of these constants bear such a relation to one another that the titration of phosphoric acid with a given alkaline solution requires exactly twice as much of the alkali when phenolphthalein is used as an indicator as when methyl orange is used as an indicator; and it is characteristic of phosphoric acid that this ratio is exactly two, a ratio which could only be precisely imitated by a mixture of two monobasic acids present in chemically equivalent quantities, the one a strong acid whose ionization constant is comparable with the primary ionization constant of phosphoric acid, and the other a vastly weaker acid whose ionization constant is comparable with the secondary ionization constant of phosphoric acid. To encounter a mixture of two monobasic acids that would fulfil the above conditions is scarcely to be taken into consideration. Hence it is safe to assume that an acid is phosphoric acid if it will exhibit accurately the described behavior toward the two indicators.

A solution of the phosphoric acid, purified as described, was therefore accurately titrated against a standard solution of sodium hydroxide using the two indicators in turn. The ratio of the two titration values obtained was 2.003 $\left(\frac{1.110}{0.554}\right)$. In spite of the fact that calibrated apparatus was used in the comparisons, the agreement between the found ratio and that theoretically required (2.000) is so far within the limits of experimental error that it may be regarded as more or less accidental.

The phosphoric acid solution was therefore assumed to be pure and was used as a standard phosphate solution in the work that follows. Its strength was calculated from the strength of the standard alkali and the titration values referred to. 1 cc. = 0.03464 gm. of $MgNH_4PO_4 \cdot 6H_2O$.

Direct Precipitation of Phosphoric Acid with Magnesia Mixture.

Eight portions of standard phosphoric acid were measured out and made alkaline with ammonia. After the addition of 10 or

20 cc. of 10 per cent ammonium molybdate,[7] the phosphoric acid was precipitated at the boiling point by the addition of magnesia mixture a drop at a time and with constant agitation, variable excesses of the reagent being used in the individual cases. The fluid was treated with one-third of its volume of concentrated ammonia and after standing over night in a corked vessel, the coarsely crystalline, heavy precipitate of magesium ammonium phosphate was filtered off. When air-dry, the weighed filters were dusted free from the magnesium ammonium phosphate and again weighed.

TABLE I.

Determination No.	Standard phosphoric acid used.	$MgNH_4PO_4.6H_2O$ obtained.	Calculated per cc.
	cc	gm.	gm.
I	9 98	0.3453	0 03459
II	10.05	0.3480	0.03462
III	9.73	0.3367	0.03460
IV	9.97	0.3457	0.03467
V	10.02	0.3474	0.03467
VI	9.88	0.3417	0.03458
VII	15.06	0.5226	0.03470
VIII	11.99	0.4142	0.03455
Mean.			0.03462
Required.			0.03464

In these experiments wide variations were made in all conditions (the excess of reagents, time of standing, etc.) and the results which are tabulated in Table I show that exactness in this respect is not necessary.

[7] Ammonium molybdate was added because without its addition the precipitate formed by magnesia mixture was flocculent and bulky and, of course, would to some extent adhere to the filter paper when dry thus entirely vitiating quantitative work. This is almost always found to be the case when a pure phosphate (ammonium phosphate) is treated with magnesia mixture. But when ammonium molybdate is first added to the ammonium phosphate solution, magnesia mixture forms a heavy sandy precipitate with no adhesive properties. It has been commonly observed that a refractory solution of ammonium phosphate will give the desired results if the phosphoric acid is first precipitated with molybdic solution and magnesia mixture added to a solution of the yellow precipitate in ammonia. This is because the yellow precipitate contains molybdic acid, $(NH_4)_3PO_4 \cdot 12MoO_3 \cdot 2HNO_3 \cdot H_2O$, and its solution in ammonia contains ammonium molybdate, $(NH_4)_3PO_4 + 12(NH_4)_2MoO_4 + 2NH_4NO_3$.

Properties of Magnesium Ammonium Phosphate.

With the material obtained in the experiments above described and in qualitative experiments it was shown that magnesium ammonium phosphate forms crystals having the composition $MgNH_4PO_4 \cdot 6H_2O$ which can be preserved unaltered for any length of time when exposed to the air. 5 molecules of its water of crystallization are easily driven off at 105° but the 6th molecule remains at 130°. The stability of the compound under ordinary conditions makes it useful as a stock material for the preparation of standard solutions of its constituents.[3] We do not know a more exact nor a more rapid method of standardizing an acid than to distill into a measured volume of it, the ammonia formed from a weighed amount of magnesium ammonium phosphate.

Contrary to the usual opinion, magnesium ammonium phosphate is very quickly converted into magnesium pyrophosphate by heating. 1 minute at bright redness is sufficient for the conversion of 250 mg. of the substance. Table II.

TABLE II

	Theoretical	Found							
		I	II	III	IV	V	VI	VII	VIII
$6\ H_2O$	44.08								
$5\ H_2O$	36.73	37.33	37.49						
$Mg_2P_2O_7$	45.38			45.22	45 30	45.20			
N	5 71						5.71	5.71	5.72

I. 0.5483 gm. lost 0.2047 gm. by heating at 105° for 1 hour, and 0.2057 gm. by heating at 115–120° for 2 hours.
II. 0.4713 gm. lost 0.1767 gm. by heating at 105° for 1 hour, and 0.1784 gm. by heating at 115–135° for 2 hours.
III. 0.2521 gm. gave 0.1140 gm. $Mg_2P_2O_7$.
IV. 0.2565 " " 0.1162 " "
V. 0.2580 " " 0.1167 " "
VI. 0.7840 " required 12.08 cc. standard acid (1 cc. = 0.00365 N).
VII. 0.6148 " " 9.63 " " "
VIII. 0.6668 " " 10.46 " " "

[3] Not for experiments in which Nessler's solution is to be used.

Influence of Ammonium Molybdate on the Quantitative Precipitation of Magnesium Ammonium Phosphate.

It has been stated that the presence of a small amount of ammonium molybdate in a phosphate solution causes the subsequent precipitation of magnesium ammonium phosphate in gross crystalline form and from the analytical data given one would conclude that the precipitation is quantitative. It is, nevertheless, desirable to make an independent test of the matter.

To measured volumes of the standard phosphate solution were added large and variable amounts of ammonium molybdate and the phosphoric acid of each solution was determined as described. The results given in Table III show that even an enormous excess of ammonium molybdate is without quantitative influence on the result.

TABLE III.

Standard phosphoric acid.	10 per cent ammonium molybdate.	Magnesium ammonium phosphate obtained.	Calculated per cc.	Percentage of theoretical.
cc.	*cc.*	*gm.*	*gm.*	*per cent*
9.72	0	0.3368	0.03464	100.0
9.42	54	0.3259	0.03460	99.9
9.84	81	0.3386	0.03441	99.3
9.91	108	0.3416	0.03447	99.5

Accuracy of the Precipitation of Ammonium Phosphomolybdate.

A test of this question seems superfluous until we reflect that the accuracy of the precipitation has always been and can only be ascertained by inference from combined processes. For the sake of completeness we made the following experiments which we think submit the matter to a sufficiently rigid test. Accurately weighed specimens of coarsely crystalline, analyzed magnesium ammonium phosphate were dissolved in nitric acid and precipitated with ammonium molybdate. The yellow precipitate was then reconverted into magnesium ammonium phosphate which was weighed. Table IV.

Oxidation of Organic Matter.

The correctness of an organic oxidation cannot, of course, be proved by an isolated test; it must be done by an end to end

TABLE IV.

Initial $MgNH_4PO_4.6H_2O$.	Magnesia mixture used.	Final $MgNH_4PO_4$ $6H_2O$.	Percentage recovery.
gm.	*cc.*	*gm.*	*per cent*
0.3078	5	0.3064	99.54
0.3150	10	0.3146	99.90
0.3121	15	0.3111	99.68
0 3087	20	0.3076	99.64

determination of the phosphorus in an organic compound and by a procedure whose remaining parts have been independently proved sound. It is, therefore, only necessary to make a correct analysis of an organic compound containing phosphorus when all the demands made of a method in the introduction to this paper will have been met. This is easy to say. We chose for the purpose adenine nucleotide, the one accessible organic compound containing phosphorus that can be purified by recrystallization to any desired extent and whose composition is independently known by a quantitative summation of its hydrolytic products. The substance was prepared from yeast nucleic acid by the method of Jones and Abt[4] and its purity was checked by a nitrogen analysis.

A weighed amount of the material (300 to 350 mg.) was "Kjeldahlized" with 10 cc. of concentrated sulfuric acid, 7.5 gm. of potassium sulfate, and 5 drops of 10 per cent copper sulfate. The product was diluted to 200 cc. as it was quantitatively transferred to a suitable short necked flask and after the addition of 4 gm. of ammonium nitrate, the phosphoric acid was precipitated at the boiling point with an excess of 4 per cent ammonium molybdate. The yellow precipitate was immediately filtered off, washed with acid ammonium nitrate solution, dissolved in ammonia, and treated at the boiling point very slowly, a drop at a time, with magnesia mixture. After the cooled material had been treated with one-third its volume of concentrated ammonia and had stood over night, the sandy, grossly crystalline precipitate of magnesium ammonium phosphate was filtered off and allowed to dry in the air. The weighed filter with its contents, held with a forceps (not with the fingers), was sharply tapped free from the non-adhesive crystalline material and after 10 minutes was weighed again.

[4] Jones, W., and Abt, A. F., *Am. J. Physiol.*, 1919–20, l, 574.

I. 0.2114 gm. required 11.15 cc. standard acid (1 cc. = 0.003641 N).
II. 0.2067 " " 10.91 " " "
III. 0.3104 " gave 0.2094 gm. $MgNH_4PO_4 \cdot 6H_2O$.
IV. 0.3307 " " 0.2216 " "

TABLE V.

	Theoretical.	Found.			
		I	II	III	IV
N	19.18	19.20	19.21		
P	8.49			8.53	8.48

Finally, it seemed desirable to test the method on a substance which contains unoxidized phosphorus and only a trace. In order to secure comparable data we analyzed a specimen of the ash-free casein described by Van Slyke and Bosworth.[5]

I. 1.0930 gm. gave 0.0628 gm. $MgNH_4PO_4 \cdot 6H_2O$.
II. 1.0306 " " 0.0605 " "

Determination No.	Phosphorus.
	per cent
I	0.72
II	0.73
Obtained by Van Slyke and Bosworth	0.71

[5] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 203.

THE REFRACTOMETRIC DETERMINATION OF SERUM PROTEINS.

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In the determination of protein content of fluids by means of the refractometer as developed by Reiss (1, 2) there are deducted from the refractive index of the serum, the refractive index of distilled water (1.3332 at 17.5°C.) and 0.00277 for the non-protein constituents, such as sodium chloride and urea, and the remainder divided by 0.00172 gives the percentage of protein with a limit of error of 0.3 per cent protein. The fair constancy of this factor Reiss attributed to the supposed constancy of the albumin-globulin ratio in the human species. However, it is difficult to see why 0.00172 should be the value of the factor since the increase in refractive index caused by 1 per cent albumin is 0.00183 and by globulin 0.00227. Since there is always some globulin present, the factor should be between 0.00183 and 0.00277, and should certainly not be less than 0.00183. One would be forced to such a conclusion by the more recent work of Heyder (3) and Rohrer (4) in Naegeli's laboratory who showed in the case of the horse and man that the refractive index of a mixture of albumin and globulin is a pure additive summation of the increments of refraction due to the percentages of albumins and globulins present, and thus disproved Schorer's (5) contention that in such mixtures the refractive index of the albumin is affected by the presence of globulins and *vice versa*, a fact which would have accounted for the low factor. Rohrer would account for this factor by the assumption that Reiss' value for the refractive index change of non-protein constituents was too high, and should be around 0.0018 instead.

It occurred to the senior author that by ultrafiltering sera through collodion tubes, the refractive index of the ultrafiltrate

minus that of distilled water would be equal to that of the filterable or non-protein constituents. Accordingly, the following procedure was adopted. The difference between the refractive indices of the serum and of its ultrafiltrate (after the refractive index of the drops coming through the filter had become constant) was used to determine the refractive index increment of the proteins. A portion of the serum was then mixed with an equal volume of saturated ammonium sulfate[1] and filtered. The refractive index of the filtrate was then equal to that of half saturated ammonium sulfate, of the non-protein constituents, and of one-half of the albumins originally present, and thus the third could be determined. The value of the albumins divided by 0.00183 was then assumed to give the percentage of albumins. The value of the albumins deducted from the value of the refractive index increment of the total proteins, gave the value of the globulins, and this divided by 0.00227 was assumed to give the percentage globulins. The total percentage calculated was then used to calculate the factor for unit percentage protein in the mixture, and was also compared with the percentage proteins as determined by the Kjeldahl method. In Table I the results are of human sera.

From this table it will be noted that the value of the refractive index increment for the non-protein constituents of human sera varies from 0.0026 to 0.0020, the mean value being 0.0022. The globulin-albumin ratio has a value varying from 12 to 34 per cent; the greater number of samples being around 30 per cent, which fact is confirmed by the observations of Rohrer and Bircher and McFarland (6). It will be noted that protein contents calculated from factors based on globulin-albumin agree fairly well with chemical analyses, the percentage deviation being relatively small in all but three cases.[2] In the next to the last column are given

[1] It was found that this procedure did not give different results from that of first diluting the serum twice and then saturating with an equal volume of ammonium sulfate. Thus in one case, 32 per cent globulin was found by the first method and 33 per cent by the other. The first was preferred as any error in reading the refractometer is only multiplied twice and not six times as in the second method.

[2] The same procedure is applicable to dog and pig sera. In the former, the refractive index increment of the non-protein constituents varied from 0.0018 to 0.0027 with an average of 0.0022, and the factor per unit per cent protein varied from 0.00195 to 0.002 with an average of 0.00198. In the latter, values of non-protein constituents were between 0.0025 and 0.0026, and the factor for per cent protein 0.00197 (B.S.N.).

TABLE I.

Refractive index of serum.	Refractive index of ultrafiltrate.	Refractive index of distilled water.	Refractive index increment of non-protein constituents.	Refractive index increment of total protein.	Globulin-albumin ratio.	Factor for 1 per cent protein.	Protein calculated.	Protein found by Kjeldahl.	Deviation of calculated from found.	Protein calculated by Reiss method.	Deviation value from found.
							per cent	per cent	per cent	per cent	per cent
1.3515	1.3355	1.3329	0.0026	0.0160	13:87	0.0019	8.46	8.51	−0.6	9.17	+ 7.7
1.3547	1.3355	1.3331	0.0024	0 0192	22:78	0.00193	9.96	9.32	+6.9	10.93	+17.3
1.3519	1.3352	1.3329	0.0023	0.0167	12:88	0.0019	8.80	8.56	+2.9	9.42	+10.0
1.3517	1.3352	1.3330	0.0022	0 0165	27:73	0.00195	8.41	8.29	+1.4	9.25	+11.6
1.3515	1.3351	1.3331	0.0020	0.0164	34:66	0 00198	8.28	8.38	−1.2	9.07	+ 8.23
1.3518	1.3352	1.3331	0.0021	0.0166	27:73	0.00195	8.50	8.43	+0.9	9.25	+ 9.73
1.3545	1.3353	1.3331	0.0022	0.0192	34:66	0.00198	9.71	9.59	+1.3	10.81	+12.7
1.3519	1.3353	1.3331	0.0022	0.0166	26:74	0.00195	8.53	8.25	+1.3	9.40	+13.9
1.3511	1.3352	1.3331	0.0021	0.0159	24:76	0.00194	8.20	8.21	−0.11	8.84	+ 7.67
1.3528	1.3353	1.3331	0.0022	0.0175	21:79	0.00193	9.10	8.74	+4.2	9.83	+12.5
1.3521	1.3353	1.3330	0.0023	0.0168	22:78	0 00193	8.71	8.52	+2.2	9.48	+11.3
1.3517	1.3350	1.3330	0.0020	0.0167	28:72	0.00195	8.56	8.09	+5.8	9.25	+14.1
1.3525	1.3351	1.3330	0.0020	0.0174	28:72	0.00196	8.88	8.21	+8.1	9.65	+17.5
1.3516	1.3351	1.3330	0 0020	0 0165	24:76	0.00194	8.51	8.03	+6.0	9.13	+13.7
1.3519	1.3355	1.3330	0.0024	0.0164	26:74	0.00195	8.43	8.29	+1.7	9.30	+12.2

the protein percentages calculated according to Reiss. That is, from the refractive index of the serum is subtracted that of water for this temperature plus 0.00277 for non-protein constituent, and the difference divided by 0.00172. It will be noticed that in all cases the percentage deviation from that found by Kjeldahl of the value calculated according to Reiss is greater than that calculated by factor actually based on globulin-albumin ratio; which factor varies from 0.0019 to 0.00198, the mean value for human sera being 0.00194. While analyses with the refractometer with a good degree of accuracy can only be obtained when the globulin-albumin ratio is known, yet it is felt that more accurate results will be obtained on the average by deducting from the refractive index of human serum that for distilled water at the temperature plus 0.0022 for non-protein constituents and dividing by 0.00194 than can be obtained by the procedure given by Reiss.

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Addendum.—The discrepancies between the percentages of protein as determined by the Kjeldahl method and as calculated from the globulin-albumin ratio and the refractive index may be explained in part by some of the facts adduced by Prof. Krogh in the "Anatomy and physiology of the capillaries" recently published by Yale University. He has shown (p. 214) that the osmotic pressure per per cent protein varies in different species, and, moreover, the protein in various fluids of the same individual may have divergent values of osmotic pressure per per cent protein (p. 265, foot-note 2). It is very probable that the refractometric increment of a per cent protein is likewise a function of the degree of dispersion of the protein as is the osmotic pressure. Work is now being done by the senior author on the effect of salts and other filterable constituents on the osmotic pressure and refractometric increment of serum proteins. (B. S. N.)

INGESTED FAT AND BODY FAT AS PRECURSORS OF THE ACETONE BODIES.*

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In a paper published in 1909 Forssner described an experiment in which he studied the effect of diets high in fat upon the excretion of the acetone bodies. For a period of about 3 weeks he took a diet consisting only of protein and fat which furnished sufficient calories for his metabolic requirements and which contained enough protein to maintain nitrogen equilibrium. He varied this basal diet in a number of ways; he changed the number of meals in which the food was taken, and the relative amounts of protein and fat eaten at the different meals; he also reduced the amount of food ingested during 24 hours, and on 1 day—the 19th of the experiment—he took no food at all. Following this day of starvation he planned to take only fat for a period of 3 days, and then repeat the starvation experiment, but rather serious symptoms resulted, and the experiment was discontinued after 1 day when nothing but fat was eaten. The results showed that the excretion of the acetone bodies in the urine (reported as "total β-hydroxybutyric acid") was fairly constant except when the fat intake was changed, and that when this was decreased the amount of β-hydroxybutyric acid also decreased, while on the day when nothing but fat was eaten a big increase in the excretion of that acid occurred.

The metabolism on each day of the experiment was such as should have caused an excretion of acetone according to the formula recently proposed by Shaffer (1922) for studying such

* A preliminary report of this work was read before the meeting of the Western New York branch of the Society for Experimental Biology and Medicine on December 16, 1922.

conditions. The average excretion of β-hydroxybutyric acid on the 11 days when the basal diet was taken was 10.6 gm., and the amounts excreted daily ranged from 7.9 to 13.7 gm.; the amount predicted by Shaffer's formula (based on the average amount of nitrogen present in the urine during this period) was 10.2 gm. The agreement when less fat and protein were eaten was not so good (for instance, on the starvation day the amount calculated was 29.2 gm. while the amount excreted was only 6.1 gm.), but in no instance were the periods long enough to establish new levels of acetone and nitrogen excretion to correspond with the changed diets.

Shaffer (1921, 1922), Wilder, Boothby, and Beeler (1922), Wilder and Winter (1922), Hubbard and Wright (1922), and Hubbard and Nicholson (1922) have recently discussed the production of the acetone bodies on the assumption that equal amounts of them were formed from the incomplete combustion of ingested fat and of fat drawn from the reserve supplies of the organism, although the last named authors called attention to slight increases in acetone excretion when fat was added to the diet of undernourished cases. In view of the experiment of Forssner further investigation into this problem seemed desirable, and the experiment described below was undertaken.

The subject used was a woman 21 years old who was 160 cm. tall and who weighed 61 kilos. Throughout the experiment she did work which was approximately equivalent to the light laboratory work done by R. S. H. studied in an earlier paper (Hubbard and Wright, 1922). Her basal metabolism was 1,500 calories.[1] In planning the diets used her caloric consumption was estimated as 1,800 calories a day, or 20 per cent more than her basal requirement. Her actual consumption was probably more than this; the figure was used to make the results comparable to those of other experiments. For 6 days the subject received a diet in which 10 per cent of the 1,800 calories was present as protein, 10 per cent as carbohydrate, and the rest as fat; the amounts of the different foods were, protein 45 gm., carbohydrate 45 gm., and fat 160 gm. These proportions are the same as those used in the basal diets in experiments already reported, and the actual amounts of the different foods correspond closely with those taken by some of

[1] Calculated from the tables gi,en in Lusk (1917), pp. 126 to 129.

the subjects already discussed (Hubbard and Wright, 1922). A sample diet for 1 day is given in the first part of Table I. From the 7th to the 9th days of the experiment the subject received the

TABLE I.

Food.		Foodstuffs.			Remarks.
Kind.	Amount.	Fat.	Protein.	Carbohydrate.	
	gm.	*gm.*	*gm.*	*gm.*	
Grapefruit	100	0	0	5.0	
Bacon	60	20.6	4.1	0	
Toast	25	0.3	2.3	13.1	
Cream	150	60.0	1.8	5 4	
Butter	45	38.2	0	0	
Asparagus	100	0.1	1.5	2.8	Sample of diets fed in first and last periods.
Peaches	100	0.1	0.5	7.7	
Tomatoes	100	0.4	0.9	3.9	
Lettuce	50	0.1	0.6	1.4	
Beans	100	0.1	1.1	3.8	
Chicken	75	5.7	13.2	0	
Eggs (2)	100	10.5	13.4	0	
Celery hearts	50	0	0.5	1.6	
Mayonnaise	18	18.0	0	0	
Cream cheese	20	6.7	5.1	0.4	
Total		160.8	45.0	45.1	
Ordered		160	45	45	
Bread	30	0.3	2.8	15.8	
Egg whites (2)	36	0	8.2	0	
Grapefruit	100	0	0	5.0	
Lettuce	50	0.1	0.6	1.4	
Celery	50	0	0.5	1.6	Sample of diets fed in second period.
Tomatoes	100	0.4	0.9	3.9	
Cottage cheese	50	1.0	10.9	4.3	
Peaches	50	0	0.2	3.8	
Skim milk	200	0.6	6.8	10.2	
Broiler	65	1.6	13.9	0	
Total		4.0	44.8	45.1	
Ordered		0	45	45	

same amount of protein and carbohydrate, but as little fat as could be furnished when ordinary foods were used. It was expected that her metabolism would be nearly the same as in the

preceding period, that the fat withdrawn from the diet would be replaced by an approximately equivalent amount of fat from the tissues, and that this fat would be burned under metabolic conditions similar to those of the first part of the experiment. A sample of the diets actually used is given in the second part of Table I. Following this period the diet used at the beginning of the experiment was resumed for 2 days.

The urine was collected daily, and was analyzed for acetone from preformed acetone plus acetoacetic acid and for acetone from β-hydroxybutyric acid by a method recently described (Hubbard, 1921). Control 24 hour specimens were also collected before and after the experiment, and were similarly analyzed. By the 5th day of the experiment it was believed that the nitrogen excretion would be approximately in equilibrium with the intake. After that time the nitrogen in the urine was determined by the micro method of Folin and Denis (1916), slightly modified to permit the use of the oxidizing and Nessler reagents described by Folin and Wu (1919).

The results obtained are given in Table II. The subject excreted less acetone than did those previously studied, but the amounts found ranged from ten to twenty times those found in the urine when the diet was normal, and must be considered as increased. The diet fed was such as should have caused no increased excretion of acetone according to Shaffer's formula, but the small increases noted can almost certainly be attributed to variations in the mixtures of foodstuffs burned at different times during the day and in different parts of the body (Shaffer, 1922). If the expected excretion is calculated by the formula proposed by Shaffer in 1921 and used by Hubbard and Wright (1922) the amounts predicted were much larger than the amounts found. The results certainly can be better explained by the formula proposed by Shaffer in 1922 than by the one proposed by him in 1921. On those days when fat was omitted from the diet the same amounts of acetone were excreted as when it was included. This seems to show that under the conditions of the experiment tissue fat and ingested fat give rise to equal amounts of the acetone bodies. The amounts of acetoacetic acid and of β-hydroxybutyric acid were also unchanged when the diet was altered. This shows that as far as the relationship between the different

acetone bodies can be regarded as a check upon the conditions under which they are formed (Marriott, 1914; Wilder, 1917) the compounds were produced under similar conditions in the two parts of the experiment. There was a negative nitrogen balance throughout, but the same amount was excreted during the time when no fat was eaten as during the other periods. There was

TABLE II.

Date.	Diet.				Urine.			
	Fat.	Protein.	Carbohydrate.	Calories.	Volume.	Total nitrogen.	Acetone.	β-Hydroxybutyric acid.
1922	*gm.*	*gm.*	*gm.*		*cc.*	*gm.*	*gm.*	*gm.*
Aug. 15–16	?	?	?	?	320		0.005	0.008
" 16–17	160	45	45	1,800	715		0.055	0.050
" 17–18	160	45	45	1,800	580		0.034	0.049
" 18–19	160	45	45	1,800	655		0.054	0.073
" 19–20	160	45	45	1,800	1,190		0.116	0.132
" 20–21	160	45	45	1,800	740		0.097	0.121
" 21–22	160	45	45	1,800	590	8 44	0.078	0.122
" 22–23	0	45	45	360	450	7.84	0.037	0.053
" 23–24	0	45	45	360	395	8.30	0 071	0.131
" 24–25	0	45	45	360	430	7.48	0.056	0.095
" 25–26	160	45	45	1,800	340	7.56	0.043	0.075
" 26–27	160	45	45	1,800	400	8 20	0.081	0.192
" 27–28	?	?	?	?				
" 28–29	?	?	?	?	620		0.005	0.010

The diets are given as they were ordered. The actual diets fed differed slightly from the figures given. See Table I for examples of the diets actually fed on 2 of the days of the experiment.

Under the heading "Acetone" the results of the determination of acetone from preformed acetone plus acetoacetic acid are given; under the heading "β-Hydroxybutyric acid" the acetone from β-hydroxybutyric acid is given. The acetone bodies are expressed in terms of acetone.

therefore no increased destruction of body protein when body fat replaced fed fat, and the fat in the two parts of the experiment was burned under conditions of nitrogen metabolism which were comparable. As further controls upon the similarity of metabolism in the two periods the blood was analyzed for cholesterol by the method of Bloor (1916), and for fat by the chemical method of Bloor (1917) and the microscopic method of Gage (1920). Samples

of blood taken before breakfast on the day before the experiment, on the last day of the first period, and on the morning following the last day of the diet low in fat showed no differences in the amounts of these lipoids present. Diets low, but not extremely low, in antiketogenic material, and high, but not extremely high, in fat do not cause changes in the amounts of fat and cholesterol present in the fasting blood when they are fed for periods as long as a week. The results also show that the experiment on the production of acetone from fed fat and tissue fat was carried out under conditions which remained practically unchanged, as far as the lipoid content of the fasting blood is concerned, throughout the period.

CONCLUSION.

The experiment shows that when diets relatively, but not extremely low, in antiketogenic material are fed the amounts of the acetone bodies excreted are the same whether the fat burned is derived from ingested or stored fat. The agreement which Shaffer (1922) found between the amounts of acetone predicted and present in starvation and in high fat feeding experiments alike seems to justify an extension of this conclusion to cases in which less antiketogenic material is included in the diet. It seems probable that the results of Forssner (1909) which are at variance with this conclusion can be explained by the short duration of the periods of inanition and reduced food intake which he studied, and that the slight increases in acetone excretion which Hubbard and Nicholson (1922) observed when fat was fed to diabetic patients were due to transitory production of that compound from the large excess of ketogenic material introduced into the organism.

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SYNTHESIS OF AMINO-ACIDS IN THE ANIMAL ORGANISM.

II. THE SYNTHESIS OF ORNITHINE IN THE BODY OF THE FOWL.

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Abderhalden (1) suggested the possibility of the synthesis in the animal body of several of the aliphatic amino-acids and thought that this would probably include cystine. He was quite certain, however, that none of the aromatic or heterocyclic amino-acids could be thus formed. He was of the opinion, moreover, that the mass action of ingested ammonia might prevent the deamination of some of the amino-acids and that these acids could then be used for the repair of the body tissues.

Certain of the aliphatic amino-acids, however, including cystine and lysine, can hardly be classed in this group, since modern nutritional experiments have shown that both of these acids are essential factors of the protein diet, and, therefore, scarcely possible of synthesis in the body. Likewise, regarding the aromatic amino-acids, the work of Ackroyd and Hopkins (2) seems to indicate that both arginine and histidine may be necessary for the promotion of growth and proper nutritional balance. At any rate, at least one of the two acids seems to be essential, for it is just possible that the organism can convert one into the other and *vice versa*.

In this connection, however, it is worthy of note that though ornithine is not in itself a primary amino-acid of the protein molecule, it, nevertheless, constitutes a very important part of the primary amino-acid arginine. Now if the ornithine fraction can be synthesized in the body, no doubt the guanidine fraction of the arginine molecule could be easily obtained from one or another of the physiologically important compounds, such as creatine or creatinine, of which guanidine forms an essential part.

It has been shown by one of us in conjunction with Shiple (3) that a human being on a non-protein diet can synthesize glycocoll or glutamine, respectively, when fed benzoic or phenylacetic acids, and that when both of these aromatic acids are fed together the organism will build both glycocoll and glutamine simultaneously for the purpose of detoxicating the foreign compounds. It is still more interesting to note that both of these amino-acids are synthesized from waste nitrogen of endogenous source, which nitrogen otherwise would have appeared in the urea fraction of the urine.

Jaffé (4) found that benzoic acid fed to birds did not combine with glycocoll as when fed to human beings or to other animals, but combined with ornithine to form dibenzoyl ornithine—which compound he called ornithuric acid. It seemed to us that this reaction might be used to determine the possibility of ornithine synthesis, and in this way to throw some light indirectly at least on the arginine problem. It was just possible, however, that ornithine would not be formed under these conditions, for Thomas (5) fed benzoic acid to hens maintained on a carbohydrate diet and failed to find ornithuric acid in the excreta. This, however, was quite to be expected since he used the whole excreta in his work, making no attempt to separate the urine from the feces. Besides he fed only a small amount of benzoic acid. It is true that after he had added edestin to the diet along with the benzoic acid he did isolate ornithuric acid from the excreta. This result he then attributed to the large amount of arginine in the edestin and concluded that ornithine could not be synthesized in the body of the fowl.

We determined, therefore, to study this point in detail, and in case we found that the fowls could not build the ornithine on a non-nitrogenous diet, we planned to add to the carbohydrate diet first inorganic nitrogen, then organic nitrogen, the latter in the form of various amino-acids other than arginine.

EXPERIMENTAL.

Large cocks were chosen, preferably those more than a year old, for these seemed to stand the strain of the feeding as well as of the operation much better than any other fowls. The chickens were provided with an artificial anus[1] according to the technique de-

[1] The surgical work connected with this problem was performed by Dr. Jesse G. M. Bullowa.

scribed by Völtz (6). This author describes the operation as a very simple one, with results so satisfactory that one bird might be used for experimental work for months. We found, however, that it was rather exceptional for such a bird to be fit for experimental purposes for more than 7 to 10 days. In three cases out of nineteen, however, we were able to employ the same bird over a period of 3 or 4 weeks.

During the experimental work the birds were placed in roomy cages and the urine and feces were collected separately. The urine of the normal chicken is a thick, ropy liquid, consisting mainly of pasty, white masses of uric acid surrounding the feces. Apparently, most of the water discharged through the ureters into the cloaca is reabsorbed from the cloaca, for the average normal chicken voids little or no liquid with the excreta. But chickens provided with the artificial anus excrete from 150 to 600 cc. of urine per day. This urine consists of two parts; a thin, serous liquid, and a pasty, white precipitate. The work, therefore, must necessarily be duplicated; that is, one set of determinations must be carried out on the liquid urine and another set made on the urinary precipitate. In general we followed the method outlined by Szalágyi and Kriwuscha (7), but in the case of the uric acid we met with so much difficulty that we finally adopted the method of Folin and Wu (8).

The following determinations were made: total nitrogen, uric acid, urea, ammonia, creatine-creatinine, free benzoic acid, and combined benzoic acid. For the determinations of free and combined benzoic acid the method of Kingsbury and Swanson (9) was used. We found considerable trouble in adapting the method to this work, but finally succeeded in obtaining sufficiently accurate results, as shown by the data obtained when benzoyl ornithine or free benzoic acid was added to the urine in order to check up our method.

The results given in Table I are taken from the work on one chicken and cover a period of about 4 weeks. The diet was practically nitrogen-free, consisting of unpolished rice, boiled potato, and water, to which was added a small amount of yeast each day. The bird's bowel was kept as free as possible from fecal matter by being thoroughly syringed each morning and evening. This was found to be very essential for the general

Days.	Total nitrogen.	Total uric acid nitrogen	Uric acid nitrogen.	Urea nitrogen.		Ammonia nitrogen.		Creatine and creatinine nitrogen.		Free benzoic acid.	Combined benzoic acid.	Total benzoic acid.	Fed.
	gm.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	gm.	gm.	
1	0.784	0.612	79.3	0.005	0.7	0.094	11.3	0.010	1.3				
2	0.764	0.491	64.2	0.005	0.6	0.094	12.3	0.029	3.7				
3	0.622	0.456	73.4	0.002	0.4	0.072	11.6	0.029	4.7				
4	0.642	0.422	65.8	0.003	0.4	0.093	14.6	0.033	4.9				
5	0.952	0.550	57.7	0.030	3.2	0.069	7.2	0.031	3.3	0.63	0.37	1.00	1 gm. benzoic acid.
6	0.897	0.526	59.8	0.072	8.0	0.108	11.8	0.029	3.5	0.40	0.51	0.91	1 " " "
7	0.584	0.265	45.4	0.053	9.1	0.144	24.7	0.018	3.2	0.30	0.41	0.72	1 " " "
8	0.405	0.232	57.4	0.030	7.6	0.033	8.3	0.009	2.2	0.00	0.23	0.23	
9	0.366	0.231	63.2	0.001	0.4	0.100	27.3	0.007	2.1				
10	0.521	0.271	52.0	0.001	0.3	0.077	14.7	0.015	2.8	0.33	0.47	0.80	1 gm. benzoic acid.
11	0.501	0.275	54.9	0.022	4.5	0.053	10.6	0.012	2.3	0.33	0.43	0.76	1 " " "
12	0.609	0.292	47.8	0.029	4.3	0.069	11.0	0.013	2.1	0.67	0.17	0.84	1 " " "
13	0.581	0.258	45.1	0.029	5.0	0.105	21.0	0.010	1.8	0.66	0.12	0.78	1 " " "
14	0.574	0.268	46.8	0.027	4.7	0.122	21.2	0.009	1.6				
15	0.541	0.308	56.9	0.013	2.5	0.072	13.2	0.015	4.0				
16	0.749	0.364	48.6	0.034	4.6	0.171	23.8	0.017	2.3				1 gm. histidine.
17	0.553	0.422	76.2	0.020	3.7	0.031	5.6	0.019	3.5				
18	0.529	0.396	74.8	0.001	0.3	0.022	4.1	0.022	4.2				
19	0.656	0.364	55.5	0.003	0.5	0.045	6.9	0.037	5.7	0.37	0.62	0.99	1 gm. benzoic acid.
20	0.880	0.456	51.8	0.020	2.3	0.097	11.0	0.040	4.5	0.55	0.38	0.95	1 " " " 2 gm. histidine.
21	1.367	0.550	40.2	0.080	5.8	0.202	14.7	0.067	4.9	0.48	0.48	0.96	1 " " " 2 " "
22	1.347	0.672	49.1	0.042	3.1	0.216	16.0	0.074	5.5	0.47	0.51	0.98	1 " " " 1.25 gm. proline.
23	1.166	0.451	38.6	0.004	0.3	0.187	16.1	0.096	8.2	0.08	0.02	0.11	
24	0.932	0.337	37.2	0.021	2.2	0.112	12.0	0.102	10.9	0.22	0.71	0.93	1 gm. benzoic acid, 1 gm. arginine.

health of the chicken as well as for the prevention of the reabsorption of nitrogenous waste material from the intestine.

On the 5th day of the experiment, after the chicken had been reduced to a stage of endogenous nitrogen metabolism, 1 gm. of benzoic acid was added to the diet. This was repeated on the 6th and 7th days, but discontinued on the 8th and 9th days. On the 4 following days, namely the 10th, 11th, 12th, and 13th days, the feeding of 1 gm. of benzoic acid per day was resumed. From the 14th to the 18th days, inclusive, no benzoic acid was fed, but 1 gm. of histidine[2] was administered on the 16th day. From the 19th to the 22nd days, inclusive, the feeding of 1 gm. of benzoic acid per day was renewed, and in addition to this, 2 gm. of histidine were given on each the 20th and 21st days, and 1.25 gm. of proline on the 22nd day. On the 23rd day nothing was fed, but on the 24th day 1 gm. of benzoic acid was given together with 1 gm. of arginine.

In order to prove that the so called "combined benzoic acid" was an ornithine derivative, the substance (ornithuric acid) was isolated from the urine according to the method of Jaffé (4), and hydrolyzed with concentrated hydrochloric acid. The benzoic acid was then recrystallized and identified by the melting point, while the ornithine was recovered and identified as the picrate.

Szalágyi and Kriwuscha (7) found that on a normal diet about 85 per cent of the total nitrogen excreted appeared in the precipitate of the chicken's urine. This was quite to be expected since about 86 per cent of the total nitrogen appears as uric acid, and the white, pasty masses were found to be almost pure uric acid. At the beginning of our experiment we found the precipitate to contain about 60 per cent of the total nitrogen, but this percentage steadily dropped as the relative amount of uric acid decreased on the carbohydrate diet. The above mentioned investigators found only 1 per cent of their total nitrogen excreted as urea, and 1.5 per cent as ammonia, while our average was 3.33 and 11.21 per cent, respectively.

The feeding of benzoic acid causes a rise in the endogenous metabolism, as is shown in the increase in total nitrogen excretion following each dose of the acid. Particularly is this exhibited by the

[2] For the histidine used in this experiment we wish to thank Dr. D. D. Van Slyke of The Rockefeller Institute.

decided rise in the urea nitrogen during the feeding period where there is an increase of 500 to 1,000 per cent over the period of rest. Though there appeared simultaneously a slight absolute increase in uric acid excretion, relatively, however, there was a decided decrease, as is shown by the drop from an average of 70 per cent for the 4 preliminary days to an average of 54.5 per cent during the first 3 days of the feeding. Again, during the second feeding period (from the 10th to the 13th days, inclusive), there was another relative decrease in the output of uric acid. This seems to indicate that the nitrogen for the amino groups in the ornithine synthesis is taken from the waste fraction of nitrogen which would otherwise appear as uric acid.

It is interesting to note that histidine and proline have little effect on the output of ornithuric acid, while, of course, arginine shows a decided tendency to add to the amount of this substance excreted. It would not seem, therefore, that histidine is very easily converted into arginine in the body of the chicken. Still more is this conclusion impressed upon one by the observation that histidine seems to contribute its nitrogen largely to the urea and ammonia fraction while arginine shows a particular tendency to excrete its guanidine nitrogen as creatine-creatinine rather than urea.

Although the fowl is able to synthesize ornithine, it is still a question whether the guanidine residue can or cannot be attached to the δ-amino group of the ornithine molecule to synthesize the latter into arginine. We are not in a position at the present time to decide this point. Still, from a nutritional standpoint it seems that such a reaction is possible, though the attacking of the problem from that angle is rather unsatisfactory due to the wide margin of uncertainty naturally involved. Nevertheless, if it can be shown that either histidine or arginine is an absolutely essential amino-acid for general nutritional purposes, and that a diet lacking these acids becomes adequate when supplemented by ornithine, one would be justified in drawing one of two conclusions; either that the organism is able to use ornithine as a foundation for the synthesis of arginine, or that ornithine itself constitutes the one essential part of the arginine molecule and that the complete synthesis of the arginine is unnecessary.

SUMMARY.

Apparently, ornithine can be synthesized from the waste uric acid nitrogen. The ingestion of amino-acids other than arginine does not seem to increase greatly the output of ornithuric acid, apparently contradictory to the theory that histidine is convertible quite easily into arginine, for ingested histidine does not augment ornithuric acid excretion while arginine does. Proline and histidine seem to increase in particular the urea output, while arginine adds to the creatine-creatinine fraction. The greater part by far of all this amino-acid nitrogen appears in the excreta of hens as uric acid nitrogen.

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STUDIES IN URIC ACID METABOLISM.

III. THE INFLUENCE OF FATS AND CARBOHYDRATES ON THE ENDOGENOUS URIC ACID ELIMINATION.

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In a former paper (1) it has been demonstrated that the excretion of endogenous uric acid in man is markedly increased by amino-acids as well as by proteins and that comparatively small quantities of amino-acids (10 gm.) are able to effect the stimulation of the output of endogenous uric acid. Since neither urea, ammonium chloride, nor sarcosine exerted any demonstrable influence on the endogenous metabolism of uric acid, the theory was advanced that the rise in uric acid excretion following ingestion of high protein diets was due to a specific stimulation of the cellular metabolism by the amino-acids, the products of the digestion of proteins. This stimulation might be occasioned by the presence of amino-acids themselves in the tissues or by their deamination products, the keto- (or hydroxy-) acids. Attention was called to certain correlations between this influence of amino-acids on uric acid elimination and their specific dynamic action and it was suggested that possibly similar chemical factors might be responsible for both of these phenomena. This theory has been recently discussed critically by Rose (2) and further evidence in support of it offered.

The purpose of the present study is to present similar data on the influence of carbohydrates and fats and their derivatives on the hourly elimination of endogenous uric acid. Previous experiments had not been considered satisfactory since in many cases

* Early experiments in connection with this problem were carried out with Dr. E. A. Doisy in the summer of 1916 and with M. S. Dunn in 1917.

the foodstuffs to be studied had been added to a diet in which other types of foods were present. Also the urines were collected in periods of 24 hours in most cases, long periods in which slight variations in the uric acid excretion might be obscured.

Four men, students or instructors, served as subjects of the experiments. The experiments with R. C. extended over a longer period of time than did the experiments with the other subjects and included a study of more types of foodstuffs. Nearly all the experiments were checked, however, by repetition on at least one other subject. However, with one exception (Table V), the experiments with subject R. C. alone are reported, in order to condense the data.

Subject R. C. was a healthy young man, 21 years of age and about 67 kilos in weight. The experiments were usually conducted on Wednesdays and Fridays. From Monday noon until the conclusion of each week's experiments, a "purine-free" diet, low in its protein content was consumed. On the evening immediately preceding the day of an experiment, a light supper was eaten and no further food was taken until the completion of the day's experiment, except the substance whose influence on uric acid excretion was to be studied.

As in other similar experiments carried out in this laboratory (1), 200 cc. of water were ingested hourly during the experimental periods in order to insure a sufficiently large volume of urine, to minimize errors due to incomplete emptying of the bladder. Since the excretion of creatinine tends to remain approximately constant from hour to hour and is not influenced by the diet, if free from creatine and creatinine, creatinine was also determined to afford an index of the completeness of the collection of the urine.

Uric acid was determined by the method of Benedict and Hitchcock and creatinine by the micro method of Folin.

Control experiments in which no food was ingested were made at frequent intervals. The results of such studies with subject R. C. are given in Table I. In confirmation of previous work in this and other laboratories (1, 3, 4) there was a tendency toward a diminished excretion of uric acid in the latter part of the morning. Also, it was noted that in the experiments during the latter part of the period (*cf.* Experiment 31, Table I; Experiment 28,

Table III; Experiments 32 and 33, Table IV), there was a slight drop in the level of uric acid excretion, probably associated with a continued low protein intake.

Ingestion of Fat.

In most of the older experiments on the effect of fat ingestion on uric acid excretion, the fat was added to a mixed diet and the urine collected over 24 hour periods. Hence it is difficult to clearly separate the various factors involved in any changes observed. Hermann (5) added 250 and 350 gm. of butter, respectively, at a single meal to a normal mixed diet (already containing 80 gm. of butter) on 2 successive days and noted the "fallende Tendenz" of uric acid excretion, but concluded that fat had no effect on uric acid elimination. Rosenfeld and Orgler (6) noted a slight increase in uric acid when 190 gm. of butter were fed to a fasting man. Horbaczewski and Kanera (7) superimposed 100 gm. of butter on a diet high in protein (daily nitrogen elimination in urine about 15 gm.) and observed a slight decrease in uric acid associated with a diminished nitrogen output, which they attributed to a weak protein-sparing action of the fat. Umeda (8) likewise found that the excretion of uric acid on a fat-rich, carbohydrate-poor diet was diminished, but attributed the effect to the acidosis occasioned by lack of carbohydrate in the diet. Macleod and Haskins (9) and others have noted an increased output of uric acid after the admin-

TABLE I

Subject: R. C. Normal controls. No food ingested. 200 cc. of water per hour.

Hour.	Experiment 1.		Experiment 3		Experiment 9		Experiment 15.		Experiment 20		Experiment 31.	
	Volume.	Uric acid	Volume	Uric acid	Volume	Uric acid	Volume	Uric acid.	Volume.	Uric acid	Volume.	Uric acid.
	cc.	*mg.*	*cc*	*mg*	*cc*	*mg.*	*cc.*	*mg.*	*cc.*	*mg.*	*cc.*	*mg.*
7–8	70	32.0	310	27 8	230	31.4	160	31.2	305	28.8	70	22.7
8–9	400	33.8	660	32.7	400	33.4	400	34.3	325	28.7	325	22.0
9–10	290	29.7	310	32.3	265	32.9	285	31 6	265	28.9	235	24.1
10–11	150	29.6	160	24 9	100	31.0	250	27.3	70	26.6	210	22.5
11–12	210	28.6	120	27.0	30	26 9	70	24.6	70	22.2	290	23.4

istration of alkali, an observation which was confirmed by Umeda. He believed that the acidosis resulting from the high fat diets caused a reduction in the alkalinity of the body fluids and probably checked the output of uric acid. This phase of the problem should be investigated further. Mendel and Stehle (3) in a study of hourly elimination could not observe any change in the excretion of uric acid after feeding butter.

In Table II are recorded the results obtained with the ingestion of cream. No change in the uric acid elimination was evident. Since it is well recognized that fat is relatively slowly digested and

TABLE II.

Subject: R. C. Influence of fat ingestion. 200 cc. of water per hour.

Hour.	Experiment 6.			Experiment 14.			Experiment 25.		
	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.
	cc.	mg.	mg.	cc.	mg.	mg.	cc.	mg.	mg.
6-7				245	32.8	72.3	93	26.9	76.3
7-8	275	35.7	61.3	395*	35.3	65.2	63*	18.5	67.5
8-9	350†	35.5	65.8	100	35.0	75.0	96	24.0	82.0
9-10	60	33.7	68.7	260	36.1	71.3	60	26.4	62.3
10-11	225	28.6	64.4	88	24.9	64.4	62	19.8	75.5
11-12	265	28.6	66.2	145	23.8	64.2	88	20.1	69.8
12-1	220	28.5	68.2	90	27.3	69.2	165	18.3	66.2
1-2							61	19.1	75.0

* 1 pint of whipping cream (30 per cent fat) at 7 a.m.

† ½ pint of whipping cream (30 per cent fat) at 8 a.m.

absorbed, it seemed advisable to continue the experiments for a longer period of time, in order to afford further opportunity for digestion and absorption of the fat, and to allow the absorbed products of digestion to exert any metabolic influence. Bloor (10) has shown that the maximum content of blood fat is reached in 4 to 6 hours after fat ingestion. In one experiment with a dog recorded by Murlin and Lusk (11), after the ingestion of fat, the heat production gradually rose until the 6th hour to a maximum and then fell slowly to the basal level, which was reached 10 hours after the fat feeding. In another animal the rise appeared earlier. In order to afford more time for any effect of the fat to be mani-

fested, Experiment 14 was continued for 6 hours, and Experiment 25 for 7 hours after the fat ingestion. No effect on uric acid excretion could be observed.

In view of these results, it was considered important to determine whether the previous ingestion of fat would in any way interfere with the stimulating action of amino-acids on uric acid output (1), either by preventing their effect or by delaying the increased uric acid elimination. In Table III, the results of the administration of glycocoll to subject R. C. are presented, results which confirm the earlier work from this laboratory. When

TABLE III.

Subject: R. C. Influence of glycocoll ingestion and of glycocoll following ingestion of fat.

Hour.	Experiment 5.			Experiment 28.		
	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.
	cc.	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*
7–8	45	23.0	63.0	63	24.2	68.4
8–9	260*	28.8	69.3	210†	23.7	68.2
9–10	95	45.0	67.4	205	22.5	65.0
10–11	360	48.2	68.2	130	23.1	66.8
11–12	175	33.6	66.8	77‡	26.4	66.8
12–1	80	28.6	65.5	255	30.1	70.0
1–2				73	23.8	65.2
2–3				60	20.0	63.8

* 10 gm. of glycocoll at 9 a.m.

† 1 pint of cream at 8 a.m.

‡ 10 gm. of glycocoll at 11 a.m.

glycocoll was administered 3 hours after fat feeding (Experiment 28, Table III), a sharp rise in the uric acid output occurred. This rise does not appear as marked as in the control experiment (Experiment 5) but it should be remembered that the glycocoll was ingested later in the morning, at an hour when the uric acid excretion tends to diminish so that the absolute rise may be more marked than appears. This experiment clearly demonstrates that the effect of amino-acids in uric acid elimination is not influenced by previous fat ingestion.

Horbaczewski and Kanera (7) and Umeda (8) have found that glycerol added to the diet caused a notable increase in uric acid

elimination. The effect was noted with glycerol, but not with glycerol combined in the fat molecule.

The results of our experiments on the effect of glycerol administration on the hourly excretion of uric acid are shown in Table IV. Ingestion of 50 gm. of glycerol resulted in an increased output of uric acid comparable to that produced by 10 gm. of glycocoll, an

TABLE IV.

Subject: R. C. Influence of ingestion of glycerol. 200 cc. of water per hour.

Hour.	Experiment 26.			Experiment 27.			Experiment 30.		
	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.
	cc.	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*
7–8	390	33.9	66.7	95	21.6	56.8	72	26.7	65.5
8–9	155*	29.9	50.5	160*	35.3	55.0	255*	29.5	63.8
9–10	93	41.5	59.4	77	39.6	52.7	100	39.6	60.7
10–11	80	37.1	55.5	28	25.6	53.7	72	30.0	60.0
11–12	190	29.9	58.9	135	27.2	58.2	250	25.9	60.6
12–1	195	22.1	61.2	330	21.2	54.3	225	23.1	65.4

Hour.	Experiment 32.			Experiment 33.		
	Volume.	Uric acid.	Creatinine.	Volume	Uric acid.	Creatinine.
	cc.	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*
7–8	135	21.2	67.5	250	23.9	65.9
8–9	140	21.2†	65.7	320‡	28.9	67.5
9–10	82	31.5	65.2	74	24.1	58.7
10–11	73	34.2	69.3	220	23.6	67.2
11–12	60	25.8	66.7	235	22.1	60.2
12–1	320	22.2	65.9	55	22.3	58.5

* 50 gm. of glycerol at 8 a.m.
† 55 gm. of glycerol at 8 a.m.
‡ 10 gm. of glycerol at 8 a.m.

increased output which, as in the case of the amino-acid, was shown in the first 3 hours after administration. The effect of 10 gm. of glycerol was negligible.

The stimulating effect of the glycerol appears more striking than that of any other substance, except the amino-acids, which we have studied. It is notable that free glycerol should produce so marked an effect, while glycerol esters in the form of fat were

without influence. Two points should be considered in this connection. The amount of glycerol fed in the form of fat could hardly have exceeded 13 gm. at the most. Also the absorption of this relatively small quantity of glycerol would presumably extend over several hours because of the slow rate of digestion of fat, and after absorption the glycerol would probably be immediately resynthesized into fat. Glycerol present in excess over that required for fat synthesis may be oxidized through the intermediary stages of glyceric aldehyde, methyl glyoxal, and pyruvic acid or converted into glucose (12, 13). In connection with the possible occurrence of pyruvic acid as an intermediary metabolite of glycerol, it is of interest to note that Gibson and Doisy (14) have recently reported an increase in the hourly excretion of uric acid following ingestion of pyruvic acid.

Carbohydrate Ingestion.

With the exception of the experiments of Smetánka (15) and Mendel and Stehle (3), the older observations on the influence of carbohydrates on uric acid metabolism are not striking. Smetánka obtained increased excretion of uric acid after the ingestion of large amounts of honey (200 to 300 gm.) and potatoes (375 to 450 gm.). He explained these results on the basis of increased activity of the liver cells in the formation of glycogen. The results of the experiments of Mendel and Stehle, in which 220 gm. of sucrose were fed, were not clean-cut. Graham and Poulton (16) suggest that carbohydrate may function in the synthesis of endogenous uric acid.

In Table V are recorded the results obtained after administration of small amounts (100 gm.) of glucose, sucrose, and lactose. Reducing sugars were present in the urines voided in the first few hours after ingestion of lactose and glucose, but not after sucrose. The experiments were uniform in their failure to demonstrate any influence of these carbohydrates in the amounts fed on uric acid excretion.

When, however, larger amounts of carbohydrates in the form of honey and glucose syrup (Karo) were administered (Tables VI and VII), more striking results were obtained. With glucose syrup, the ingestion of amounts less than 200 gm. exerted little

influence on uric acid output, while with 300 gm. the effect was marked. Similar results were obtained with honey. With amounts less than 200 gm., the results were variable and not striking. 200 gm. or more, however, caused a slight rise in uric

TABLE V.

Subject: M.D.* Influence of ingestion of small quantities of glucose, sucrose, and lactose. 200 cc. of water per hour.

Hour.	Experiment 36.			Experiment 27.			Experiment 28.		
	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.
	cc.	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*
6-7				25	16.2	50.0			
7-8	21	16.8	52.5	36	16.1	52.1	80	16.4	51.7
8-9	58	22.4	58.0	22†	19.7	55.0	202	19.0	61.2
9-10	55‡	17.4	54.2	20	18.3	54.4	61†	15.4	53.0
10-11	46	15.2	56.1	214	20.5	57.9	89	19.0	58.5
11-12	166	17.4	59.8	190	17.7	59.1	93	17.6	55.3
12-1	366	22.3	60.7	45	15.1	53.5	160	17.9	55.5

Hour.	Experiment 29.			Experiment 30.		
	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.
	cc.	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*
6-7				105	17.6	59.9
7-8	65	19.9	52.0	273	18.4	55.1
8-9	243	22.6	62.9	277§	17.9	60.1
9-10	213§	19.6	58.6	252	16.1	57.4
10-11	303	23.0	62.7	271	16.0	58.8
11-12	103	17.6	55.0	106	14.4	56.1
12-1	127	19.2	61.7	110	13.8	57.8

* The fasting controls on this subject are given in the earlier paper of Lewis, Dunn, and Doisy (1).

† 100 gm. of sucrose at 8 a.m.

‡ 100 gm. of glucose at 9 a.m.

§ 100 gm. of lactose at 8 a.m. (Experiment 30) and 9 a.m. (Experiment 29), respectively.

acid excretion. With neither carbohydrate material was the increased elimination as marked as with amino-acids or glycerol.

It is of interest to compare the effects of the different foodstuffs studied from the quantitative standpoint. The influence of the amino-acids (1) is most marked, 10 gm. of glycocoll causing as

sharp an increase in uric acid output as 300 gm. of glucose syrup. It is to be noted that types of foodstuffs other than proteins and their derivatives exerted an influence on uric acid excretion only when very large quantities were consumed, that is, when the organism was overwhelmed by the absorbed substance, as evidenced by the appearance of reducing sugar in the urine in the case of

TABLE VI.

Subject: R. C. Influence of ingestion of various amounts of honey.* 200 cc. of water per hour.

Hour.	Experiment 22.			Experiment 21.			Experiment 8.		
	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.
	cc.	mg.	mg.	cc.	mg.	mg.	cc.	mg.	mg
7-8	350	23.7	65.7	220	28.6	76.5	160	32.9	63.6
8-9	170	22.2	70.9	245	24.6	74.1	270	32.7	70.5
9-10	245	25.7	68.5	270	31.2	75.2	110	36.8	70.0
10-11	345	27.1	64.6	225	35.7	71.2	325	36.1	60.5
11-12	125	24.6	68.5	59	28.3	73.2	240	40.8	69.8
12-1	205	23.5	66.2	225	26.7	76.8	105	34.6	68.0

Hour.	Experiment 23.			Experiment 24.			Experiment 7.		
	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.	Volume.	Uric acid.	Creatinine.
	cc.	mg.	mg.	cc.	mg.	mg.	cc.	mg.	mg.
7-8	480	29.3	64.3	375	22.6	71.2	145	31.2	57.2
8-9	210	25.1	71.0	58	27.7	69.5	205	29.3	68.8
9-10	125	27.0	69.5	150	24.2	70.4	80	34.3	62.7
10-11	225	31.3	73.2	290	35.4	72.5	92	32.1	69.7
11-12	315	27.8	63.0	350	34.5	61.7	450	35.5	66.5
12-1	265	22.6	64.3	275	30.5	72.5	150	31.8	59.1

* Honey was eaten at 8 a.m. in the following amounts: Experiment 22, 110 gm.; Experiment 21, 120 gm.; Experiments 8, 23, and 24, 200 gm.; and Experiment 7, 215 gm.

the carbohydrates. The influence on uric acid excretion runs roughly parallel to the phenomena of specific dynamic action. Proteins stimulate heat production greatly and presumably by a chemical mechanism different from that which functions in the case of stimulation by fats and carbohydrates (17). The stimulation of energy metabolism by carbohydrates is less than that produced by proteins and the effect of fat is less marked than that

of carbohydrates. Lusk (17) considers that the effect of carbohydrates and fats on heat production is due to the metabolism of plethora, the flooding of the cells with oxidizable fragments of carbohydrate or fat. Similarly, the effect of carbohydrate on uric acid excretion is apparent only after the ingestion of such large amounts that the organism cannot metabolize them normally

TABLE VII

Subject: R. C. Influence of ingestion of various amounts of glucose syrup.* 200 cc. of water per hour.

Hour	Experiment 17			Experiment 16			Experiment 10		
	Volume	Uric acid	Creatinine	Volume	Uric acid	Creatinine	Volume	Uric acid	Creatinine
	cc	mg	mg	cc	mg	mg	cc	mg	mg
7-8	260	35.4	66.7	330	31.0	78.6	260	34.6	60.7
8-9	255	31.5	71.6	130	30.1	62.1	370	31.1	67.2
9-10	170	30.6	72.6	125	34.8	70.0	215	32.2	72.6
10-11	310	32.0	69.8	240	38.8	70.8	300	29.8	66.7
11-12	160	29.2	67.2	390	29.4	58.0	170	26.7	67.4
12-1	200	26.1	69.5	73	31.8	61.5	165	25.4	69.3

Hour	Experiment 11			Experiment 12		
	Volume	Uric acid	Creatinine	Volume	Uric acid	Creatinine
	cc	mg	mg	cc	mg	mg
7-8	60	28.5	52.3	375	31.9	70.7
8-9	185	30.6	74.2	260	34.0	71.3
9-10	235	44.2	74.2	115	34.3	69.5
10-11	105	31.3	67.8	35	40.3	69.7
11-12	50	27.4	70.2	35	24.2	65.8
12-1	300	27.2	69.2	320	26.2	70.8

* Glucose syrup (Karo) was eaten at 8 a.m. in the following amounts: Experiment 17, 125 gm.; Experiment 16, 190 gm., Experiment 10, 210 gm.; Experiment 11, 310 gm.; and Experiment 12, 320 gm.

and traces of sugar appear in the urine. Relatively small amounts of amino-acids, on the other hand, increase uric acid excretion rapidly and effectively.

It would seem that the results presented do not offer any evidence against the theory of endogenous uric acid metabolism previously suggested (1); namely, that one of the factors at least in the rise of uric acid excretion following ingestion of various

purine-free foodstuffs is a general stimulating action upon cellular metabolism and consequently upon nuclear metabolism by the foodstuffs or their products of digestion or catabolism.

SUMMARY.

1. Fat (cream) in amounts up to approximately 135 gm. did not influence the hourly rate of elimination of uric acid in the fasting individual.

2. Glycerol caused a marked increase in the hourly uric acid excretion. This effect was observed during the 2nd and 3rd hours after its ingestion. Amounts of glycerol equivalent to the glycerol contained in the fat fed exerted no demonstrable influence on the uric acid output.

3. The stimulating action of amino-acids (glycocoll) on uric acid excretion was not affected by the ingestion of fat previous to the administration of the glycocoll.

4. Lactose, sucrose, and glucose in moderate amounts (100 gm.) did not influence uric acid elimination. Honey and commercial glucose syrup in amounts greater than 200 gm. caused an increase in the elimination of uric acid, but the effect was neither so constant nor so marked as with amino-acids or glycerol.

5. It is believed that these results support the theory that the increases in the output of endogenous uric acid, following the ingestion of non-purine foodstuffs are due, in part at least, to a general stimulation of cellular metabolism, occasioned by the presence of the foodstuffs or their products of catabolism in the cells.

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STUDIES ON YEAST.

V. THE VITAMINE B CONTENT OF YEAST.*

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(Received for publication, December 27, 1922.)

In previous communications from this laboratory by Fulmer, Nelson, and Sherwood (1) it was found that yeast can grow and reproduce for years in a medium containing only known constituents, indicating, therefore, that neither the hypothetical bios nor vitamine B need be present in the solution in order that growth proceed. These same investigators also showed that the yeast growth method, which was first employed by Williams (2), for the estimation of water-soluble B, is not only unreliable but under no circumstances can it be used for the quantitative estimation of vitamine B in our food materials, and any data obtained by this method have no significance, even though in many cases the amount of vitamine B obtained through the yeast method runs parallel to the results secured by the use of animals. Fulmer, Nelson, and Sherwood (1), furthermore, developed a synthetic medium free from all unknown factors, and this medium is the best synthetic medium that has thus far come to the attention of the author; and it is likewise the best which can be made from the constituents employed. Since yeast was supposed to contain relatively large amounts of vitamine B, and since this substance is not necessary for the growth of the yeast cell, a test of the yeast grown on the synthetic medium should reveal whether or not this organism has the capacity to synthesize this complex from simple constituents of known structure. Subsequently, Nelson, Fulmer, and Cessna (3) of this laboratory proved that yeast does

* This paper forms part of a thesis which will be submitted to the graduate faculty of the Iowa State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

synthesize vitamine B. They grew yeast in a synthetic medium; namely, medium F, inoculating this medium with yeast that had been previously repeatedly transferred, so that the amount of original constituents—either of the medium or the original yeast—was so small as to be negligible. Yeast grown upon such a medium caused a resumption of the growth of rats, which had been fed upon a diet lacking water-soluble B until all growth had ceased. Shortly after the appearance of this article Harden and Zilva (4) published some work on the capacity of yeast to synthesize vitamine B. The data were not conclusive, but some evidence was given that certain yeasts at least can synthesize this unknown dietary factor. In a recent article, MacDonald (5) comes to the conclusion that of five species of yeast which were tried all have the capacity to synthesize the water-soluble B vitamine. Recently, while this work was in the final stages of preparation, there appeared in this Journal an article on yeast as the sole source of vitamine B by Kennedy and Palmer (6). These investigators were unable to confirm the view held by many that yeast is an unusually rich source of vitamine B, and they were unable to secure normal growth and reproduction when yeast was employed as the sole source of this particular vitamine. Likewise, Evans and Bishop (7) state in a recent communication that they have not succeeded in obtaining normal reproduction in rats with yeast as the sole source of vitamine B and have postulated that perhaps another vitamine besides those already recognized may be necessary for the production of healthy young.

The object of the experiments recorded in this paper was to determine the amount of vitamine B in yeast grown upon wort and also in a synthetic medium. It would then be possible to compare the vitamine content of the two yeasts and determine whether or not the composition of the medium will influence the amount of vitamine the cell is able to synthesize. Data are also presented to show that yeast not only synthesizes the growth-promoting vitamine B, but likewise it has the capacity to produce the antineuritic vitamine.

EXPERIMENTAL AND DISCUSSION.

Our general procedure consisted in bringing about a suspension of growth in rats on a vitamine B deficient diet and subsequently

feeding various amounts of yeast to determine the minimum amount necessary to bring about a resumption of growth. We have, furthermore, determined the influence of various amounts of yeast on growth and reproduction. The ration fed the animals consisted of purified casein 18 per cent, salt mixture (8) 5 per cent and in other cases 3.7 per cent, filtered butter fat 5 per cent, and dextrin to 100 per cent. The casein was extracted with distilled water, acidified with acetic acid. Even though the water was changed at least once a day and often several times, nevertheless, it required as long as 4 to 5 weeks to eliminate the vitamine completely. A better, or at least a more rapid method, to remove the vitamine is to extract the casein by means of 95 per cent alcohol, using large percolators equipped with a siphon arrangement similar to a Soxhlet extractor. No casein was ever employed in these experiments which had not been previously tested in order to insure the complete absence of vitamine B, in so far as that is possible by feeding experiments.

Chart 1 shows the results obtained by adding 1.5 per cent of air-dried *Saccharomyces cerevisiæ* (Fleischmann's Race F). Up to the point marked x on the curve the animals had received a basal ration containing no vitamine B. At this point 1.5 per cent of the above yeast was added. This yeast contained no filler; in other words, it consisted of pure yeast cells. A marked response in growth resulted, but the growth was by no means normal. The female on this ration did not produce young although it was apparently from external appearances in good condition and had ample time to do so.

Chart 2 illustrates the results obtained by incorporating 2 per cent of the same yeast employed in Chart 1. The growth curves are perhaps slightly better than those obtained using 1.5 per cent of yeast. The curves approximate the normal and yet no young were produced. We have not been able to obtain young in any of our animals by the use of either 1.5 or 2 per cent of yeast in the diet.

Chart 3 shows what might be expected when the ration contains 2.5 per cent of air-dried yeast. The typical growth curves are about normal. Female 215 had a litter of four young. One of these young died in 2 weeks time and appeared in a very poor condition. The second likewise appeared to be suffering from mal-

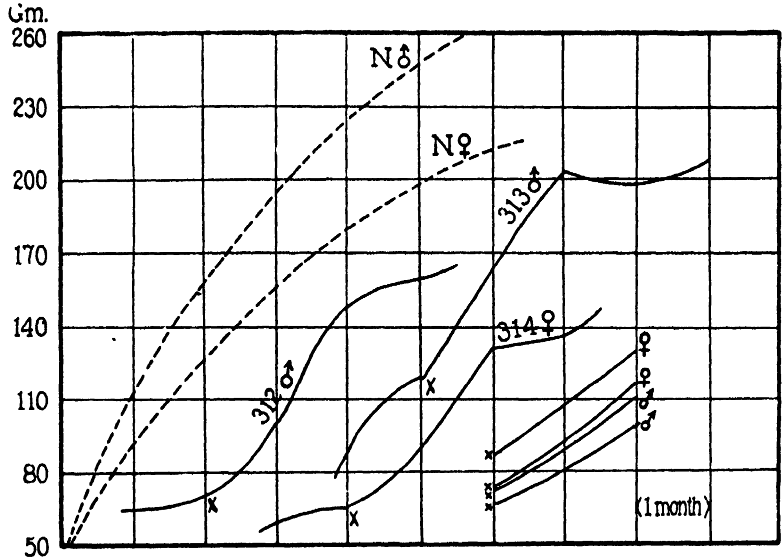

Chart 1. Curves of growth of rats receiving 1.5 per cent of yeast (*Saccharomyces cerevisiæ*).

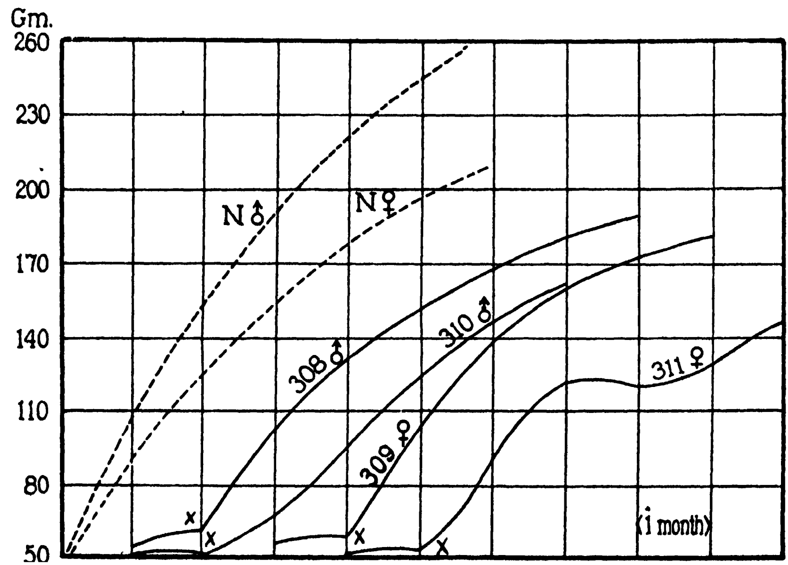

Chart 2. Curves of growth of animals receiving 2 per cent of air-dried yeast (*Saccharomyces cerevisiæ*).

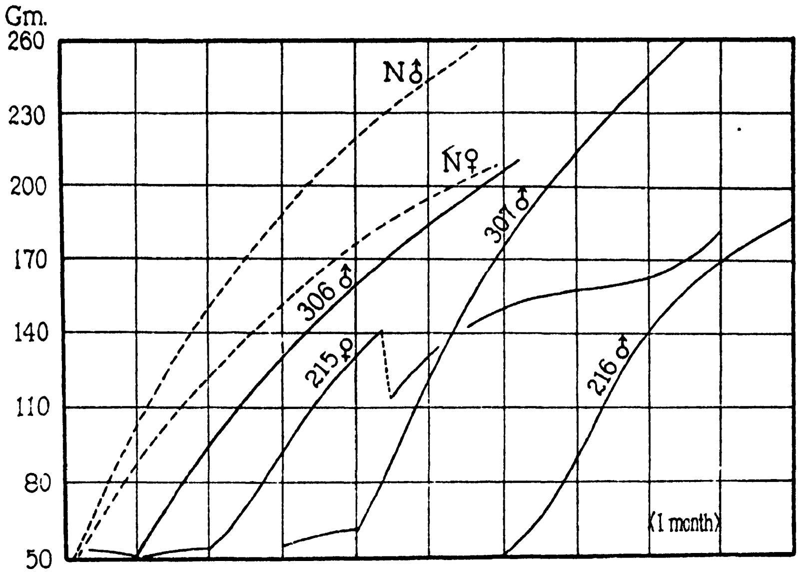

CHART 3. Growth curves of rats on a synthetic diet in which the sole source of vitamine B consisted of 2.5 per cent of air-dried yeast.

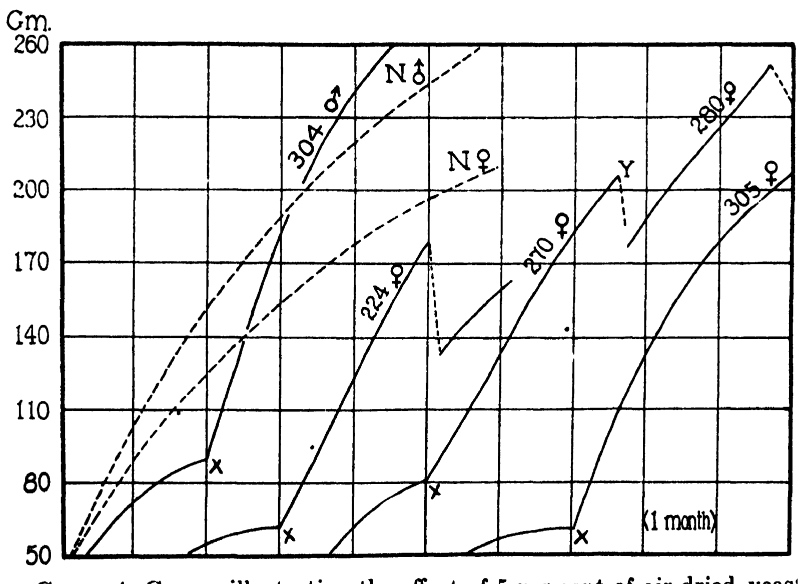

CHART 4. Curves illustrating the effect of 5 per cent of air-dried yeast in the diet.

nutrition and died the following week. The third survived 4 weeks, but shortly before the time of its death looked very emaciated, and it was far below normal in weight. The fourth rat lived and grew to maturity. Its curve of growth is given in Chart 3, No. 216. The rate of growth of this animal as judged by the curve falls just below the normal. It now weighs 180 gm. and appears in a fairly good condition. During the early stages of its growth, shortly after weaning, this rat just maintained its weight for several weeks. This portion of the curve is not represented in the chart. 2.5 per cent of *Saccharomyces cerevisiæ* permits of normal growth to maturity and even suffices for reproduction and the weaning and rearing of some of the young, but even though this be true reproduction is not what we would call normal.

Chart 4 gives the results obtained by using 5 per cent of air-dried *Saccharomyces cerevisiæ*. The animals all grew at the normal, and better than the normal, rate. Two of the females on this ration have had young. Female 224 has produced a litter of three, and they were exceptionally fine young at birth. One died at the end of the 2nd week. It looked very well for a week or so, but later appeared in an emaciated condition, and ceased to grow. The other two were vigorous young at birth but at weaning time failed to make the gains they should. Although weaned they are now below normal weight and do not look as well as normal rats should. We have observed in our studies on yeast that the females may produce exceptionally fine young and the failure of reproduction manifests itself only at or shortly before the weaning time, when the animals either cease to grow or do so very slowly. No. 270 produced one litter at the point marked Y on the curve, but she did not take care of them. Rat 280 has produced a litter of young. The young are a little over 2 weeks old and normal in weight. This animal, however, was a mature animal when placed on the ration. 5 per cent of air-dried *Saccharomyces cerevisiæ*, judged by a considerable number of experiments, allows of growth at the normal, and better than the normal, rate. It also permits of reproduction and the rearing of the young although the young do not develop as normal young should.

Charts 5, 6, and 7 show the results obtained by the use of undried yeast in place of the air-dried product. Chart 5 illustrates the fact that 3 per cent of natural undried yeast does not furnish

enough vitamine B for normal growth. This amount corresponds to 1.02 per cent of dried yeast.

Chart 6 shows the results obtained by the use of a diet containing 5 per cent of undried yeast corresponding to 1.74 per cent of air-dried solids. Growth to maturity resulted at the normal, and even better than the normal, rate. Female 232 had one litter of young, but they were destroyed. These results show the marked influence of so mild a treatment as air-drying upon the vitamine B potency of yeast. It is remarkable that this vitamine should be so stable toward heat and yet so susceptible to air-drying. The animals in Chart 2, receiving 2 per cent of air-dried yeast, did not respond as well as those in Chart 6. From numerous experiments that have been conducted in this laboratory, we are of the opinion that the undried yeast is more potent than the air-dried. But even though the animals grew to maturity at the normal rate reproduction was not normal, nor has it been in any of the animals receiving this amount of yeast.

Chart 7 shows the results obtained by the use of 6 per cent of undried yeast corresponding to 2.04 per cent of dried substance. The rats made rapid growth. Female 246 had three young but all of them died in an emaciated condition without having been weaned. The young were in very good condition and normal in size at birth. We have not succeeded in obtaining normal reproduction with this amount of yeast in any of our animals.

It is evident from these experiments that drying the yeast effects the destruction of a part of the vitamine. 2.5 per cent of air-dried yeast allows growth to maturity at the normal rate, with reproduction and rearing of a part of the young. 5 per cent of yeast permits growth at even better than the normal rate with reproduction and rearing of the young, but the young do not develop as normal young should. Experiments now in progress in our laboratory will answer the question whether it is possible to rear healthy young at all on yeast as the sole source of vitamine B, but some time must elapse before a final answer can be given. Although we may know what are the necessary dietary factors required for growth and reproduction, we do not know the concentrations of these various factors which will produce the best results. That a cell will not develop normally, without taking into consideration the concentration of the substances in which

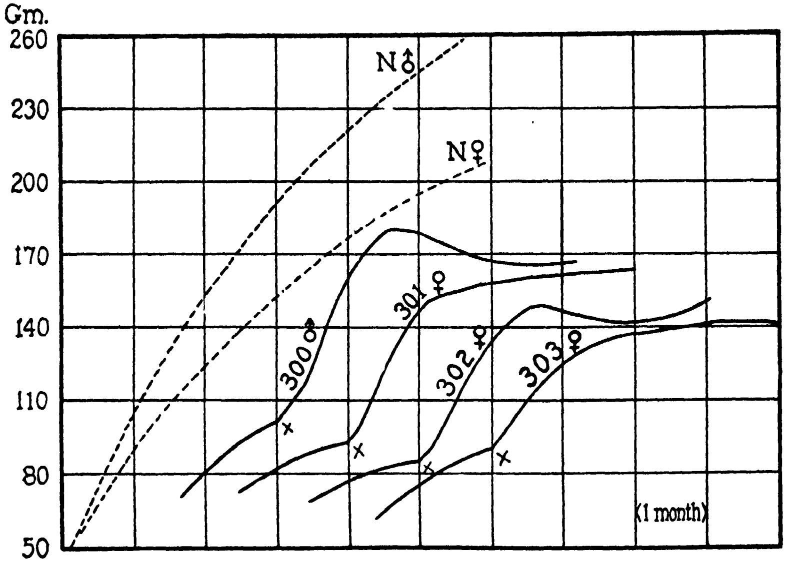

Chart 5. Curves of growth of rats which received their sole source of vitamine B from 3 per cent of undried yeast. This amount of yeast corresponds to 1 per cent of air-dried solids.

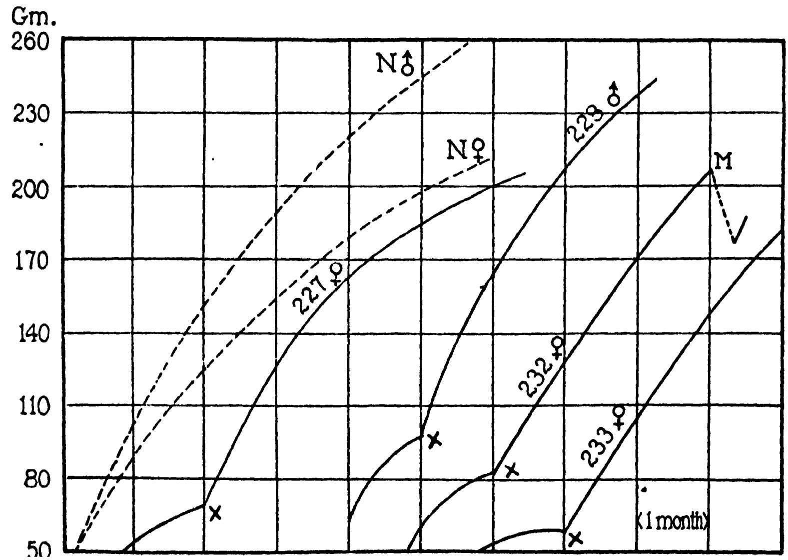

Chart 6. Growth curves of animals receiving a diet containing 5 per cent of undried yeast equivalent to 1.74 per cent of air-dried solids.

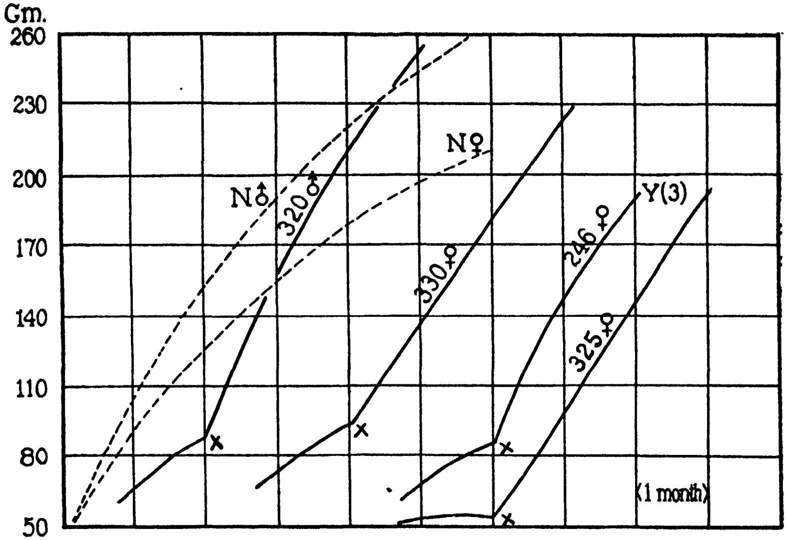

CHART 7. Growth curves illustrating the effect of 6 per cent of undried yeast in the diet. This amount corresponds to about 2 per cent of air-dried solids.

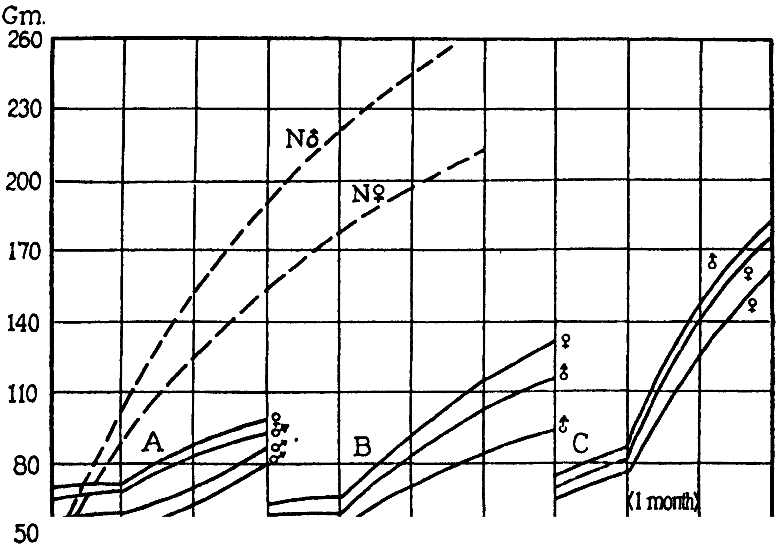

CHART 8. Curves of growth of animals fed yeast grown on a synthetic medium.

Lot A received 2.5 per cent of this yeast in the diet.

Lot B received 3 per cent in the ration.

Lot C received 4 per cent.

it is immersed, is very well illustrated by the work of Fulmer, Nelson, and Sherwood (1) of this laboratory. Yeast is very sensitive to the concentration of the various components which comprise the medium. There is an optimum or minimum concentration for each of the constituents of the medium.

Of particular interest in this connection is the comparative amounts of vitamine B in *Saccharomyces cerevisiæ* grown in wort and that grown in a synthetic medium. Considerable quantities of the latter yeast were grown in medium F, having the following composition: 100 cc. contained 0.188 gm. of ammonium chloride, 0.100 gm. of calcium chloride, 0.100 gm. of dipotassium phosphate, 0.04 gm. of precipitated calcium carbonate, 0.60 gm. of dextrin, and 10 gm. of cane-sugar. This medium must be kept at 30°C. in order to insure optimum results. The effect of adding the yeast grown on Medium F is illustrated in Chart 8. In order to save space, the results of only a few of the animals at certain definite levels are recorded. The addition of 2.5 per cent of *Saccharomyces cerevisiæ* grown on a synthetic medium produced only slow growth. When 3 per cent of this yeast was incorporated in the diet, much better growth resulted; but the growth is not normal and is not as good as when 2 per cent of this type of yeast grown upon wort was used. Data not recorded here show that 3.5 per cent is not sufficient for normal growth for a period of over 3 months. When 4 per cent of this yeast is added to the diet, normal growth occurs over a period of 2 months. We have not tested the yeast on animals for a longer period, due to the extreme difficulty of obtaining a sufficient quantity. It is, therefore, evident that although yeast, which has grown upon a synthetic medium synthesizes vitamine B, it does not contain as much of this vitamine as yeast grown in wort.

Does Yeast Synthesize the Antineuritic Vitamine?

In a recent number of this Journal Eijkman, van Hoogenhuijze, and Derks (9) published an article in which they came to the conclusion that yeast grown upon a synthetic medium is unable to cure polyneuritis in pigeons. In view of the fact that Nelson, Fulmer, and Cessna (3) of this laboratory showed that yeast can synthesize the growth-promoting or water-soluble B vitamine Eijkman and his associates are of the opinion that what is known

as vitamine B is in reality two vitamines; the one having to do with the cure of polyneuritis, and which can, therefore, be designated the antineuritic vitamine, and the other being concerned with growth can consequently be called the growth-promoting vitamine. Eijkman, van Hoogenhuijze, and Derks cultivated *Saccharomyces* isolated from baker's yeast, in a synthetic medium containing only inorganic salts and cane-sugar which they claimed was suitable for the growth of yeast, and when they fed this product to pigeons suffering from polyneuritis, it failed to establish a cure. They likewise call attention to an experiment performed previously, in which cooked, polished rice, to which Chinese rice yeast was added, failed to have any, or at least comparatively little, antineuritic potency. They noticed, furthermore, that the above species of baker's yeast when cultivated at the same temperature, namely, 27°C., in an aqueous extract of rice polishings after washing with physiological saline solution in order to remove any adhering portions of the medium, was potent in curing polyneuritis in pigeons. These same investigators likewise heated a portion of this extract of rice polishings in the autoclave at 120°C. which they claim destroys the antineuritic factor, and then grew yeast in this solution. The yeast so obtained was rich in the antineuritic vitamine while the solution which was filtered off from the yeast was found to be inactive. They also tried another strain of *Saccharomyces* which was obtained from beer yeast, and used as their synthetic medium the inorganic constituents found in beer-wort. The results were the same as before. No antineuritic vitamine was formed by the yeast grown on this synthetic medium, but when grown on beer-wort it was decidedly potent.

Eijkman, van Hoogenhuijze, and Derks say in this connection: "It seems, therefore, that yeast not only takes eventually its antineuritic factor as such from the culture medium but that it is not even capable of synthesizing the vitamine unless the medium contains at least the products of decomposition of the vitamine by heating." In view of the fact that Fulmer, Nelson, and Cessna (3) of this laboratory have demonstrated that yeast can synthesize water-soluble B, a fact which appears contradictory to their work upon pigeons, they are of the opinion that vitamine B and the antineuritic vitamine are not identical.

The yeast which was used for testing the antineuritic vitamine was cultivated in the same medium employed for the growth of the yeast which was given to rats to test for the growth-promoting power. Yeast grows much more slowly upon a medium such as this than it does upon wort, and it is, therefore, a considerable task to prepare a sufficient quantity for feeding and curative experiments. Not only is it a difficult task to prepare such large quantities in a synthetic medium, but it must also be remembered that yeast so grown must be carefully guarded from contamination, for if this species of yeast did not have the capacity to synthesize vitamine B, other varieties might possess that power and, of course, such contamination would vitiate the results. The synthetic medium used by the author is by no means identical with that employed by Eijkman, van Hoogenhuijze, and Derks, and it may be that the character of the synthetic medium will influence the synthesis of water-soluble B by the cell.

The pigeons used for the production of polyneuritis were full grown birds. The first bird came down with the acute form of the disease in 19 days on a polished rice diet. As expected, the appetite of this animal fell off greatly after a week or so and, in order to insure an adequate food allowance, the bird was forcefully fed. The pigeon was not fed the synthetic yeast addition until it manifested all the symptoms of polyneuritis, such as the characteristic posture of the head and the total inability to use the legs. At this point the air-dried yeast, which had been grown on the medium given above, was fed by mouth and within 19 hours the animal's posture was perfectly normal except that it was very weak in the limbs. Without giving any more yeast the pigeon got up and walked in a perfectly normal manner 4 hours later. The amount of yeast used for the cure of this bird was not exactly determined, but approximately 3 gm. of air-dried yeast grown upon the synthetic medium were given.

Pigeon 2 came down with all the typical symptoms of polyneuritis in 20 days. It was fed 3 gm. of moist synthetic yeast, containing approximately 1 per cent of air-dried solids. A complete cure was established in 15 hours time.

Pigeon 3 showed all the symptoms of the disease in 16 days. 2 gm. of dried synthetic yeast were then administered by mouth, and in 14 hours the bird was walking about the cage, and, although still weak, there was no question but that a cure had been effected.

Pigeon 4 had typical polyneuritis at the end of 24 days. It was given 1 gm. of yeast grown upon a synthetic medium, but died during the night. Whether this amount of yeast contained too small an amount of the antineuritic vitamine, or whether the animal was in too low a physical condition, we are unable to state.

Pigeon 5 showed the disease on a synthetic ration, complete in every way except for vitamine B, in 32 days. This animal received 3 gm. of undried yeast and a cure was effected in 24 hours.

Pigeon 6 also showed typical symptoms of polyneuritis in 32 days. The diet consisted of purified casein 18, salts 5, filtered butter fat 5, and dextrin to 100 per cent. 3 gm. of undried yeast grown upon a synthetic medium cured the animal in 24 hours.

The writer desires to thank Professors V. E. Nelson and E. I. Fulmer for suggesting this problem and for advice during the progress of the work.

Thanks are also due The Fleischmann Company for generously supplying us with the large quantities of pure yeast necessary for this and other research work in connection with the dietary properties of yeast.

SUMMARY.

1. The vitamine B potency of yeast (*Saccharomyces cerevisiæ*, Race F) has been determined. 2.5 per cent of this yeast is sufficient for growth at the normal rate. Reproduction is possible at this level, but it is perhaps not normal, although young may be brought to maturity. 5 per cent of this yeast allows growth at the normal, and even better than normal, rate. Normal young have been obtained and weaned. Shortly before weaning time, the young on this ration do not develop as normal young should.

2. Drying of yeast (*Saccharomyces cerevisiæ*, Race F) destroys some of the vitamine.

3. *Saccharomyces cerevisiæ* grown in a synthetic medium is not as rich in vitamine B as that grown in wort.

4. *Saccharomyces cerevisiæ* not only synthesizes the growth-promoting vitamine, but the antineuritic vitamine as well.

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VITAMINE B.

I. A MODIFIED TECHNIQUE IN THE USE OF THE RAT FOR DETERMINATIONS OF VITAMINE B.*

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(Received for publication, January 22, 1923.

Until the time arrives when there will be available chemical tests for quantitative and qualitative analysis of vitamine, physiological tests will have to be used. Of these, two have been used in various laboratories, *viz.* (*a*) the increments of growth resulting when the vitamine unknowns are added to an otherwise satisfactory ration of a small laboratory animal such as the rat, and (*b*) the increase in weight or numbers of micro organisms when grown with and without the addition of the vitamine unknown to a standard nutrient media. Of these two, the former alone has survived the test of time and experience; the latter having been discarded, at least temporarily, because micro biologists have come to an acute realization of the fact that the unknowns of the nutritive requirements of fungi and bacteria are too numerous; whenever a vitamine-containing solution is added, the impurities contained therein may have quite as much of an effect as the vitamine itself. Besides this, there remains the possibility, as pointed out by Emmett and Stockholm (1) and by Funk and Dubin (2), that the vitamine requirements of micro organisms may be of a different nature from that of the rat. Yet even when using the rat for all comparative work, variations in technique in different laboratories, and even in the same laboratory, unintentionally and unknowingly may be of such a nature that entirely uncomparable results are obtained.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

A number of possibilities immediately suggest themselves. In the first place, the basal food constituent may not be sufficiently purified from vitamine B to give minimum growth. Here, in ordinary basal rations as usually used, there is need of consideration being given only to the dextrin or starch and the casein or other proteins that may be used. Fats, the salt mixtures, and agar as roughage, are free from vitamine B and, therefore, need not be considered. Starch or its product, dextrin, is also low in vitamine B as indicated in numerous feeding trials, a resultant no doubt of the fact that starch is liberated by comminution of the grain in a water-soaked condition and recovered by sedimentation from flowing water suspension. We have, nevertheless, felt the necessity in some of our work of insuring a special low vitamine B content and have used for this purpose rice starch treated with alkali in the form of sodium carbonate. The sodium carbonate was subsequently removed by thorough lixiviation with water acidified with acetic acid and then distilled water for a week, changing the water daily. We have assumed that this treatment was efficacious. With the large amount of starch used in low fat rations, about 70 per cent, even a very low vitamine content of the starch may assume considerable significance.

The protein factor is also important. Fortunately, evidence has been furnished by Osborne and Mendel (3) that casein, which is used probably more than any other protein as a constituent of rat rations, has remarkably little affinity for vitamine B so that even in its crude commercial form it does not support much growth in rations otherwise satisfactory. We have always made it a point to test our crude product to make certain that it did not contain any abnormally large amounts of vitamine and then have subjected it to an additional purification as follows: 7 kilos of the crude ground casein are poured on a large piece of muslin in a tub of 75 liters capacity. Tap water is then run in, acetic acid to 0.1 per cent added, and the casein worked up thoroughly with the hands. When well mixed, the edges of the cloth are gathered and tied with a string. The acidified water is changed twice daily —the casein being worked up thoroughly by hand to expose new surfaces each time—for 5½ days. For the last five washings distilled water is used instead of tap water. At the termination of operations the casein is thoroughly rinsed with distilled water,

then drained, and dried in a steam oven for 3 to 4 days. Such casein is free from vitamine B as far as we have been able to determine.

In 1911 Osborne and Mendel (4) published their pioneer nutrition work with rats which has become classic, not because of a realization of the object of their experiment—the satisfactory determination of the comparative nutritive value of proteins—but because they pointed out the feasibility of using the growth of the rat as a criterion of the value of rations. Among other things they pointed out the fact that when rats were not performing well on certain rations improvement resulted when they were given access to their feces as a supplement. As the existence of vitamines was not known at the time, the significance of this, though appreciated, was not interpretable; yet it pointed out how vitally certain factors might be influencing the character of the final results.

In 1918 when carrying out some preliminary experiments on the requisite composition of salt mixtures used with so called synthetic diets, we took into account the fact that the excreted mineral substances might play an important part in supplementing the mineral ration in coprophagistic rats. We, accordingly, placed all our rats in cages provided with a false screen bottom 3 to 4 meshes, preferably the former, to the inch. Much to our surprise we got nutritive failure which was caused not by lack of insufficient minerals but by lack of sufficient vitamine B.

Since these results were obtained, we have paid especial attention to the tendency of rats to consume their excreta when placed on deficient diets, and have come to the conclusion that many of the determinations of vitamine B recorded in the literature are worthless. Our reasons for coming to this conclusion are brought out in the following experiments.

EXPERIMENTAL.

The experiments here recorded were not carried out with specially purified materials, because we were primarily concerned with results of comparative value; yet our basal ration has shown itself to be surprisingly low in vitamine B. Only one out of eight rats weighing from 45 to 60 gm. was able to live 46 days on this ration. The dextrin was prepared from ordinary corn-starch

as previously described (5); the wheat embryo, when used, was extracted in a large Soxhlet for 6 hours with ether; and the casein was purified as usual.

Our first results, obtained in 1918, are recorded in Charts 1 and 2. Chart 1 shows how four rats when kept on screens while being fed a synthetic ration, after 3 to 4 weeks barely maintained their weights. On such a ration fairly satisfactory growth had repeatedly been secured in other experiments although it may be well to call attention to a fact, of which we have long been cognizant, that up to the present time, none of the so called synthetic rations recorded in the literature are what they have been assumed

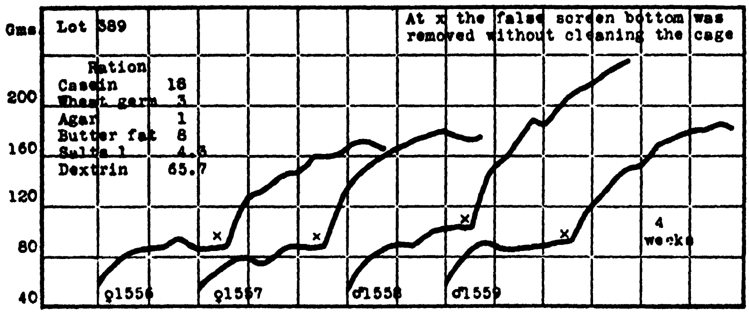

CHART 1. When rats are kept in cages provided with false screen bottoms, a ration, entirely satisfactory when the rats are kept on shavings, is no longer able to maintain normal growth. With removal of the screens, thereby giving the animals access to their excreta, growth is promptly resumed.

to be; they are not able to take the place of mixtures of salts with naturally occurring foodstuffs. The animals seemed to be entirely comfortable on the screens and it did not seem probable that the resumption of growth which occurred with the removal of the screens, thus giving them access to their accumulated excreta, was due to anything but the change of diet. With the removal of the screens feces were promptly consumed by all the rats. The ration was not changed.

In Chart 2 are shown growth curves which bring out the probable cause of the failure of growth. Lot 428 shows the growth obtained when the rats were kept on shavings. As they were fed according to the group system they were confined in a

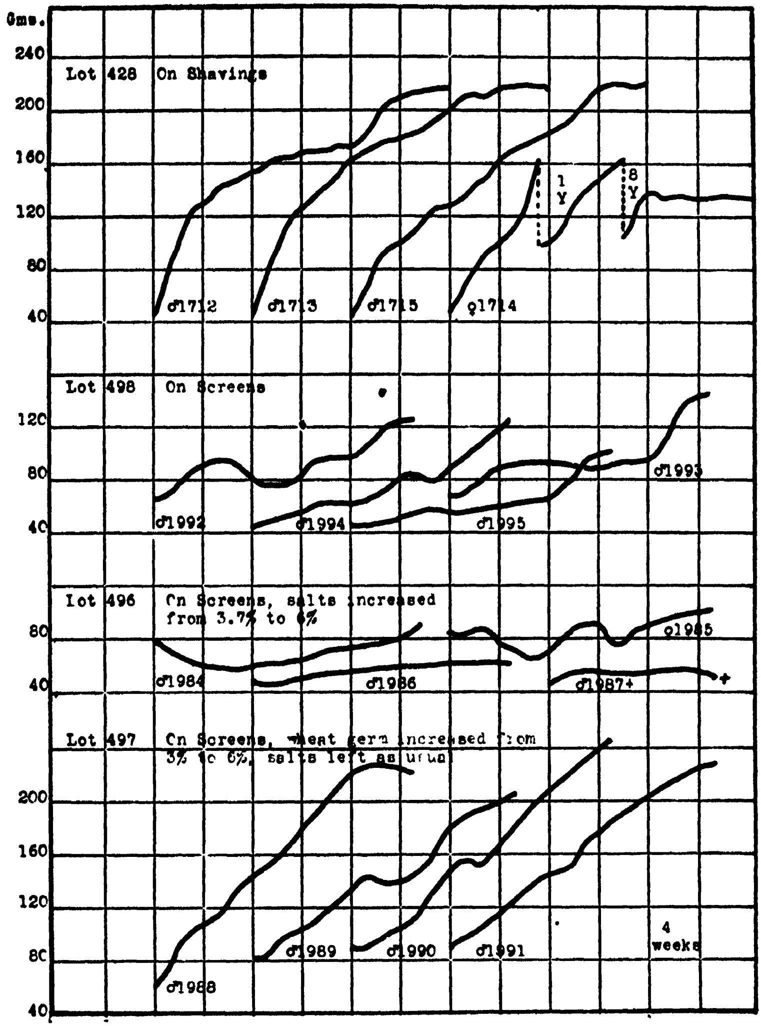

CHART 2. Growth is far inferior when the rats are kept on screens, Lot 498, than when kept on shavings, Lot 428. Apparently this is due to the fact that the rats are unable to supplement their diet with vitamine B voided with their excreta; additional salts did not improve the growth, in fact depressed it, Lot 496, but additional vitamine as wheat germ caused decided improvement, Lot 497.

rather large cage measuring 2 feet square by 20 inches high. With such a large floor space the cages do not become filthy rapidly and the bedding remains dry. For this reason they were not cleaned oftener than at intervals of several weeks and excreta for consumption were generally available.

The rations consisted of casein 18, agar 2, wheat germ 3, butter fat 5, salts 3.7, and dextrin to make 100 parts, unless otherwise indicated. The salts were added as salt mixture 1 (5) except in Lot 428 when salt mixture 32 (5) was used. As they contain the same inorganic elements in the same amounts, varying only in the manner of combination and a larger amount of lactic acid in salt mixture 1, it seems safe to assume them of equivalent value.

As the curves show, growth was long continued and far superior to what was observed when the rats were kept on screens, as shown in comparison of Lot 498, Chart 2 with Lot 389, Chart 1. This occurred in spite of the fact that the ration carried 0.6 per cent less of the salt mixture.

In Lot 496 where the salt mixture was increased to 6 per cent of the ration with the butter fat at 8 per cent, bare maintenance was secured. Salts were apparently not the limiting factors. This is supported by the growth resulting in Lot 497 where the rats were kept on screens with the salts still at a 3.7 per cent level and the butter fat at 8 per cent, but the wheat embryo increased from 3 to 6 per cent. From these records it appears that the main dietary supplement which rats obtain from their excreta is vitamine B and if the comparative amounts of growth can be used as criteria of the source of the vitamine, at least one-half of the vitamine was obtained from the excreta when the rats were kept on shavings. This indicates a tremendous loss of vitamine in the physiological economy of the animal, but our data do not indicate whether this represents vitamine unassimilated and excreted with the feces or vitamine which has served its purpose without being destroyed and then subsequently voided with the urine and absorbed by the feces. That urine (6) contains the vitamine is, however, well known.

Our recent experiments have sought to establish more definitely the importance which is to be attached to fecal consumption as a supplement to a restricted diet. We now recognize it as a matter of sufficient moment to warrant putting a considerable proportion

of our animals on screens, but even this does not entirely solve the problem, when the group system of feeding is used, as some rats are so alert in their quest for an adjuvant to their restricted diet that they will seize the feces of their companions as soon as excreted. We are inclined to believe, however, that the amount thus obtained is a factor of but little importance in determining the final result.

Chart 3 shows the comparative results obtained when rats were kept on screens or shavings at different levels of yeast intake ranging from 0.5 to 8 per cent in a basal ration consisting of casein 18; salts 32, 4; agar 2; cod liver oil 1.5; and dextrin to 100. On the basal ration alone, growth was continued for less than 2 weeks when the rats were kept on shavings—the largest increment in this time being 22 gm.—then rapid failure set in. When kept on screens, growth was maintained for 1 week, or less, the largest increment being 9 gm.; then maintenance obtained for 1 week, after which rapid failure resulted. The yeast bearing rations show that from two to three times as much yeast is required in the ration as a source of vitamine B when the rats are kept on screens than when they are kept on shavings.

Chart 4 represents the first of a series dealing with the vitamine B content of grains. If our results with rats on screens are reliable it appears questionable if our grains are provided with such a large excess of this vitamine, in comparison with the rats' requirements for maximum growth, as has been assumed. We, ourselves (7), in the past have accepted the fact that 15 to 20 per cent of our grains probably furnished enough of this dietary essential; although in our fat-soluble vitamine studies when we used white corn as a medium for introducing vitamine B, we used 40 parts per 100 of ration and kept the rats on shavings.

In the grain series casein was added to all the rations as an additional source of protein, such an amount of casein being added that the protein content of the ration was kept at approximately 18 per cent. Cod liver oil to the extent of 1.5 per cent was always added as the source of fat-soluble vitamine and 4 per cent of a complete salt mixture, salt mixture 32, was used to furnish plenty of the inorganic elements. The rest of the ration, not taken up by the grain expressed in parts per 100, was furnished as added dextrin.

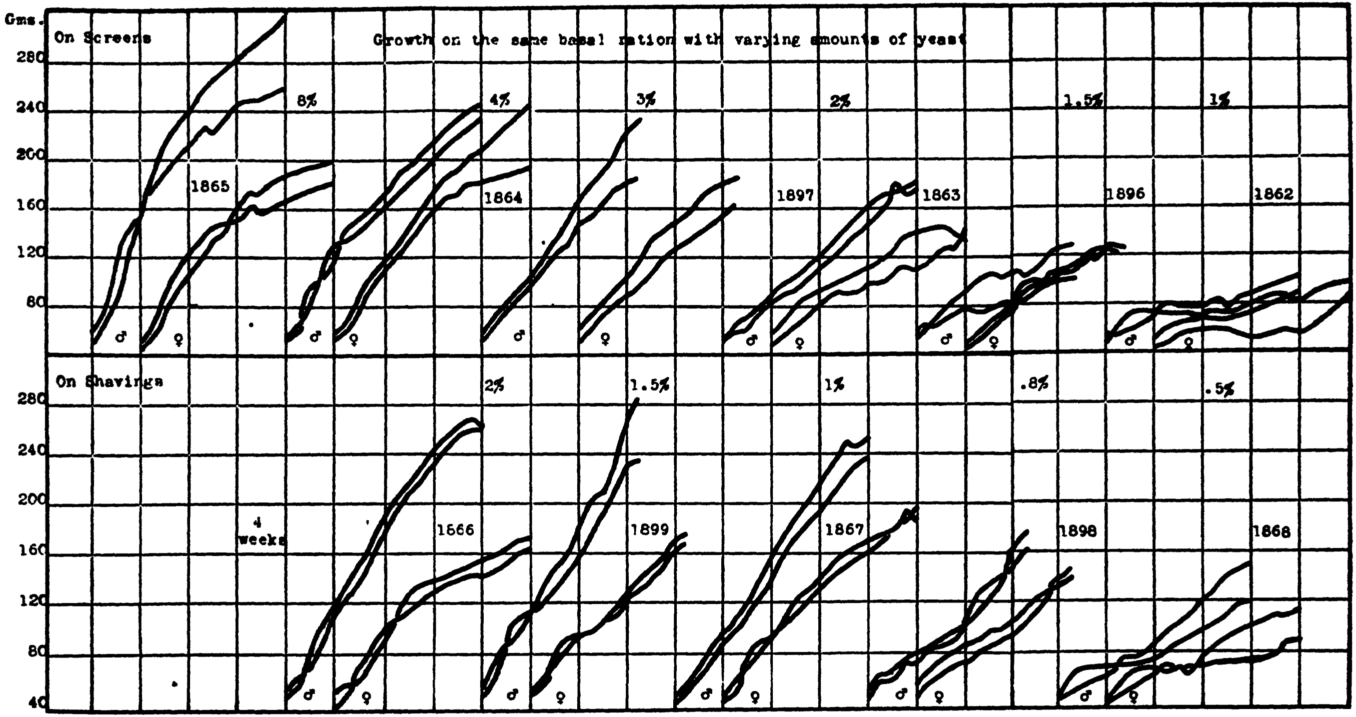

CHART 3. On a ration of casein 18; salt mixture 32, 4; agar 2; cod liver oil 1.5; with yeast varying in amount from 0.5 to 8 per cent; and dextrin to 100 the above curves of growth show that it takes from two to three times as much vitamine B to produce normal growth when rats are kept on screens than when kept on shavings.

Chart 4 shows that maximum growth was obtained when somewhere between 60 and 80 per cent of yellow corn was contained in the ration—even as much as 40 per cent was insufficient when supplementation of vitamine in the ration by the ingestion of excreta was prevented. 30 per cent of corn with access to excreta in this

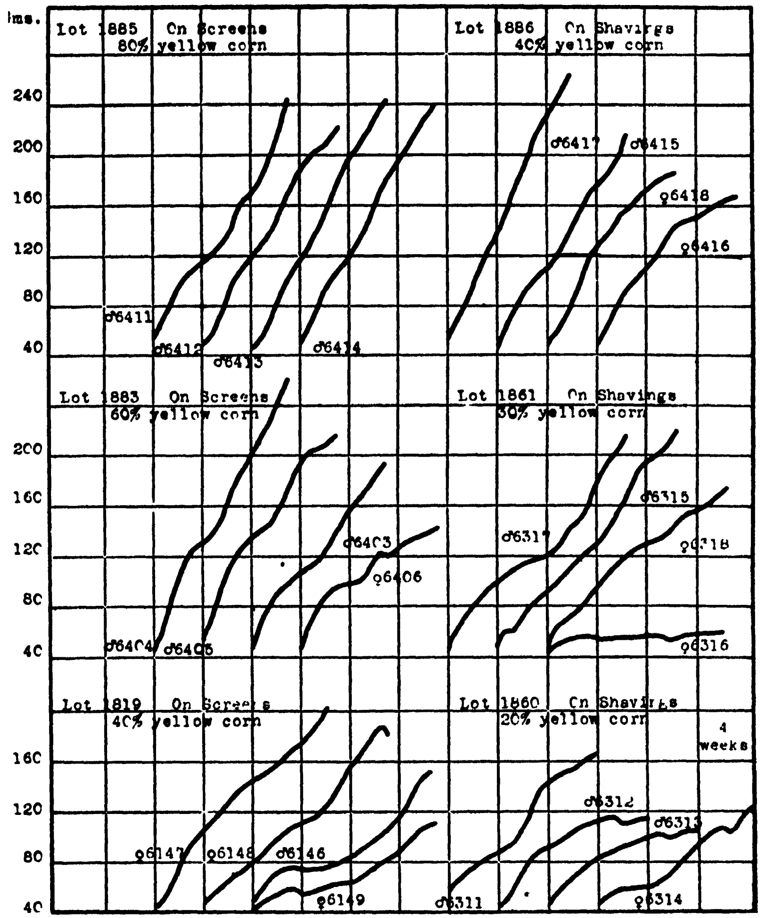

Chart 4. On a ration containing variable amounts of corn; casein to make a total protein content of 18 per cent; salt mixture 32, 4; cod liver oil 1.5; with dextrin to make 100, approximately twice as much corn is required to furnish a sufficiency of vitamine B when the rats are kept on screens than when kept on shavings.

case also failed to give normal results, a fact probably to be explained by a failure of the rats to consume enough feces. This is the disturbing feature when control over the fecal consumption is not maintained; some rats will supplement their ration early in life and liberally; others will not; and again when the cages are cleaned daily, sufficient supplementation may not always be possible. One rat of this group was obviously entirely abnormal. The quantitative relations in vitamine requirement of rats when kept on screens and when not, as supplied by corn, is brought out better in Chart 5. 10 per cent of corn when the rats were kept on

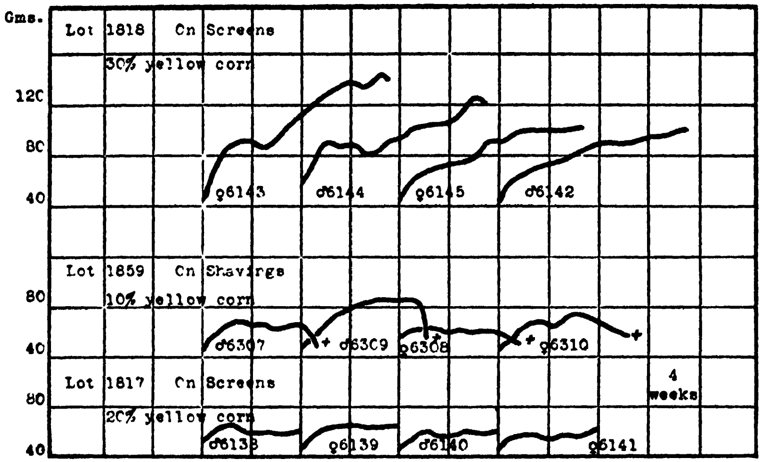

CHART 5. 10 per cent of the ration as yellow corn fed to rats on shavings gave better results than 20 per cent, though poorer than 30 per cent, when they were kept on screens.

shavings was better than 20 per cent, though poorer than 30 per cent, when kept on screens. The ratio of required vitamine content of the ration here also appears to be somewhat in excess of the ratio of 2 to 1.

Chart 6 shows the amount of growth obtained with whole ground oats as a source of vitamine B in an otherwise vitamine B-poor ration. At all levels of oat intake casein was added to make a total of 18 per cent protein; 1.5 per cent of cod liver oil was added for fat-soluble vitamine and 4 per cent of our complete salt mixture, salt mixture 32, for mineral elements. Growth on the 10 per

cent and even on the 20 per cent level was poor; even the 40 per cent level did not provide for normal growth. At this latter level the amount of growth is fairly comparable with that obtained on 40 per cent of corn.

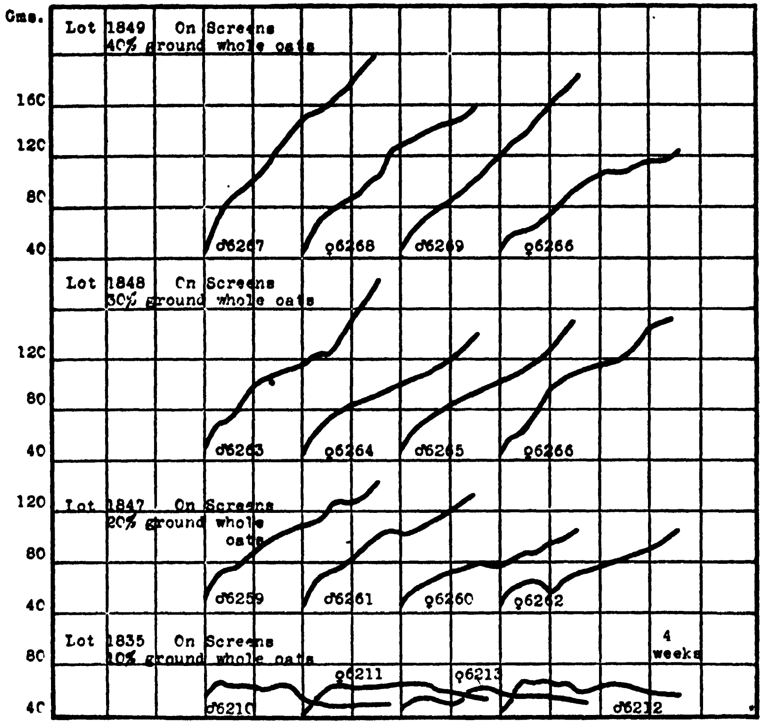

CHART 6. The rat requires more than 40 per cent of its ration as ground whole oats if the oats are to serve as the only source of vitamine B.

SUMMARY.

When rats are prevented from supplementing their diet by the consumption of excreta the vitamine B content of the experimental rations must be at least twice as high. This narrows down the margin of safety in the ability of our ordinary grains to furnish the rat with a sufficiency of the vitamine for maximum growth; it appears that in the neighorhood of 60 per cent of the ration must be grain to accomplish this purpose.

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VITAMINE B.

II. STORAGE OF VITAMINE B BY THE RAT.*

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From what has been presented in the previous paper (1) it appears that the vitamine B content of foods is not used by the animal with the greatest economy. At least, the rat when on a vitamine deficient diet is able to supplement his legitimate ration advantageously by a reingestion of the vitamine eliminated with the excreta. This together with the fact that lack of vitamine B results in failure of function of the gastrointestinal tract, thereby tending to prevent the intake of the very food which when ingested in sufficient amount might cover the vitamine requirements, lends added importance to consideration of what safeguards the animal body possesses when confronted with a vitamine deficiency. Storage of it in the tissues for emergency use as obtains in the case of carbohydrate and fat for energy, calcium and phosphorus in the bones for growth, bone repair, and lactation, or even as in the case of protein, the less essential organs yielding their constituents to those upon which the most urgent need is imposed in varied functional capacities, is worthy of consideration. Evidence on this matter for vitamine B is not directly available in the literature, but from the relatively slight variability of initial growth on rations low in this vitamine, the fact is suggested that the animal has little if any ability to store it. We have submitted the matter to experimental inquiry at the present time because rats when kept on screens show such uniformity of growth that we felt warranted in expecting decisive results.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

EXPERIMENTAL.

The experiments were carried out with the usual technique of this laboratory, making use of the group system of feeding our animals—usually two males and two females being kept in one group. They were fed and watered daily and kept under hygienic conditions in cages 2 feet square provided with false screen bottoms 3 meshes to the inch. Their low vitamine ration was composed of casein 18 (1), agar 2, salt mixture 32 (2) 4, cod liver oil 1, and dextrin (2) 75—all of the usual grade used in this laboratory and tested especially for value in the desired capacities in which they were expected to function. After being placed on the experimental ration the animals were weighed weekly until death.

Age and Weight in Relation to Vitamine B Storage.

In much of the literature dealing with vitamine determinations whether of the fat-soluble vitamine or the water-soluble vitamine as made by rat feeding experiments, little attention has been paid to the age or even the size of the rat as long as the animal was of such a degree of immaturity that considerable growth could still be made. This is not in conformity with the best experimental methods as we have come to recognize them, because it fails to take into consideration the possibility of stored reserves of the vitamine in question; it assumes that vitamine requirements are the same irrespective of age or size; and where dealing with degree of failure of maintenance it assumes that the same amount of vitamine is required in this rôle.

There are other variations in technique which are worthy of analysis in attempting to improve the quantitative factors of nutrition work where growth is used as the criterion of the amount of vitamine present. In fat-soluble vitamine analysis perfection of the quantitative side of the experimentation is hampered because the response of the animal is so varied; growth may be inhibited directly because of failure to consume food on account of blindness, on account of respiratory trouble, or directly on account of interference with cellular development of the tissues most concerned in weight increase in spite of adequate food consumption; and naturally upon improvement of the diet recupera-

tion with resumption of growth ensues to a varying degree. In addition there also enters the fact that what we speak of as the fat-soluble vitamine may in reality consist of a number of vitamines which may vary in their proportionate occurrence and thus modify the end-result. This, however, is true of all vitamine fractions as now known irrespective of type. With the water-soluble vitamine the syndrome resultant from a deficiency is not so varied. Invariably the animal reduces its food consumption and ceases to grow. With the addition of sufficient vitamine to the ration, improvement is usually very prompt and continued.

In the first series of experiments designed to bring out storage relations, four litters of rats after being reared on a ration of yellow corn 76, linseed oil meal 16, crude casein 5, ground alfalfa 2, sodium chloride 0.5, and calcium carbonate 0.5, with milk *ad libitum*, were placed on the described vitamine B-free ration at various ages. The first lot was started at an age of approximately 3 weeks, the next at 4, the next at 5, and the last at 6 weeks. Actually, two rats in each lot were exactly the given age, one was 1 day older and one, 4 days older. In each lot one rat was taken from each litter and as the rats differed in age by only 4 days fair uniformity in age and parentage among the groups was obtained.

The results are shown in Table I and Chart 1. They indicate that the rat, when kept on a ration of grains and milk—eminently satisfactory for growth and reproduction—does not store much of the water-soluble vitamine up to the time of weaning, or even in the 3 weeks following. All the rats were able to maintain life for approximately 50 days irrespective of age, but with the outstanding difference that the older the rat the greater the loss in weight before death resulted. The time when this loss in weight began varied among the lots, beginning approximately after 2 weeks in the rats 3 and 6 weeks old, and after 1 week in the rats 4 and 5 weeks old. Osborne and Mendel in a recent publication (3) have discussed the relative vitamine requirements of rats at various stages of immaturity, and experimentally from the amount of growth resulting on the same vitamine intake have concluded that older rats require more of the vitamine. Our data suggest that this is also true when considered from the standpoint of maintenance of weight. We believe these relations to be worthy

TABLE I.

Lot.	Age.	Maintenance of life.	Gain in weight.	Loss in weight from initial weight.	Loss in weight from maximum weight.
	wks.	*days*	*gm.*	*gm.*	*gm.*
1783	3	53	10	4	14
1784	4	50	17	22	39
1785	5	49	15	38	53
1786	6	50	9	44	54

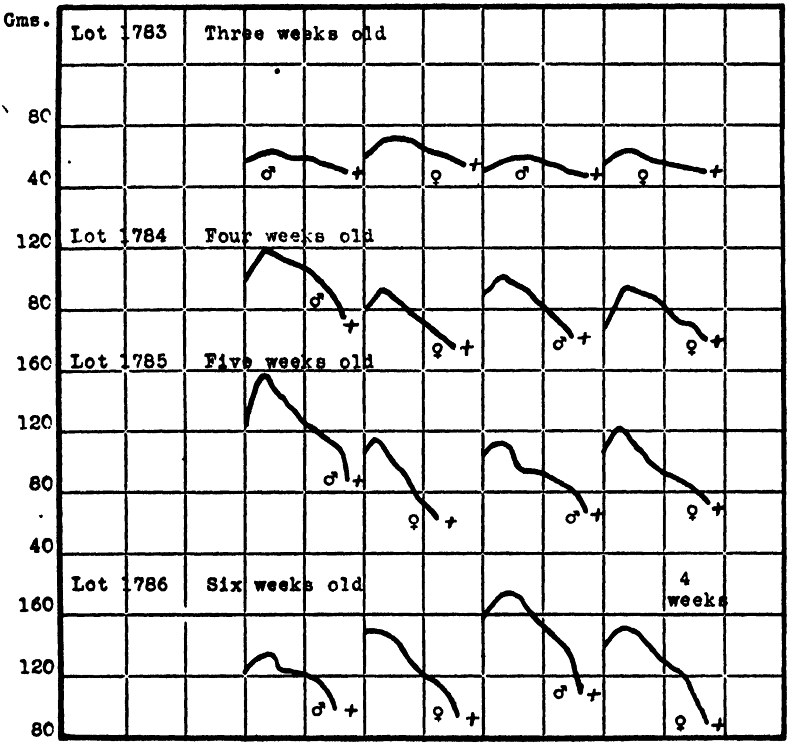

CHART 1. The effect of age on maintenance in young rats when put on a ration low in vitamine B.

of further analysis using the special precautions of keeping the rats on screens and feeding them a ration free from vitamine B. In many of these rats extreme spasticity and convulsions typical of lack of vitamine B were observed, leaving no doubt as to the immediate cause of failure.

Diet in Its Relation to Vitamine B Storage.

In the second series of experiments an attempt was made to determine the effect of variations in the vitamine B content of the diet, as consumed by the rats before being put on the experimental ration. The possibility remained that the rats of the preceding series failed to show storage of vitamine even when kept on a good ration for a prolonged period because the vitamine intake was not sufficiently large. We accordingly segregated eight pregnant females from our stock colony and reduced their young to six in number for uniformity's sake and then fed them rations of varying vitamine content as shown in Table II. The vitamine B content was varied in these rations by substitution of 10 to 20 parts of corn by brewer's yeast of which approximately 4 parts

TABLE II

	Ration XI	Ration XII	Ration XIII
Yellow corn	73	63	53
Brewers' yeast	0	10	20
Linseed oil meal	15	15	15
Crude casein	8	8	8
Cod liver oil	2	2	2
Sodium chloride	0 5	0 5	0.5
Calcium carbonate	1 5	1.5	1.5

were equal in vitamine content to 60 parts of grain as shown by actual test. This would mean that Ration XII contained, roughly, at least twice as much, and Ration XIII contained at least four times as much vitamine B as Ration XI. Two females were fed on Ration XI, three on Ration XII, and three on Ration XIII. All three rations were readily consumed and maintained, the females in excellent condition while rearing their young.

When the young were from 23 to 25 days old and were weighing from 45 to 67 gm., nine lots were put on the described ration, low in vitamine B, four rats in a lot, and each lot on screens. 1 week later two lots of four rats each which had in the meantime been kept, respectively, on Rations XII and XIII only, that is, removed from their mothers, were also started on this ration. At this time these rats weighed from 82 to 99 gm. The results

obtained with the rats started at 23 to 25 days of age are shown in Chart 2 and Table III. They indicate surprisingly little variation

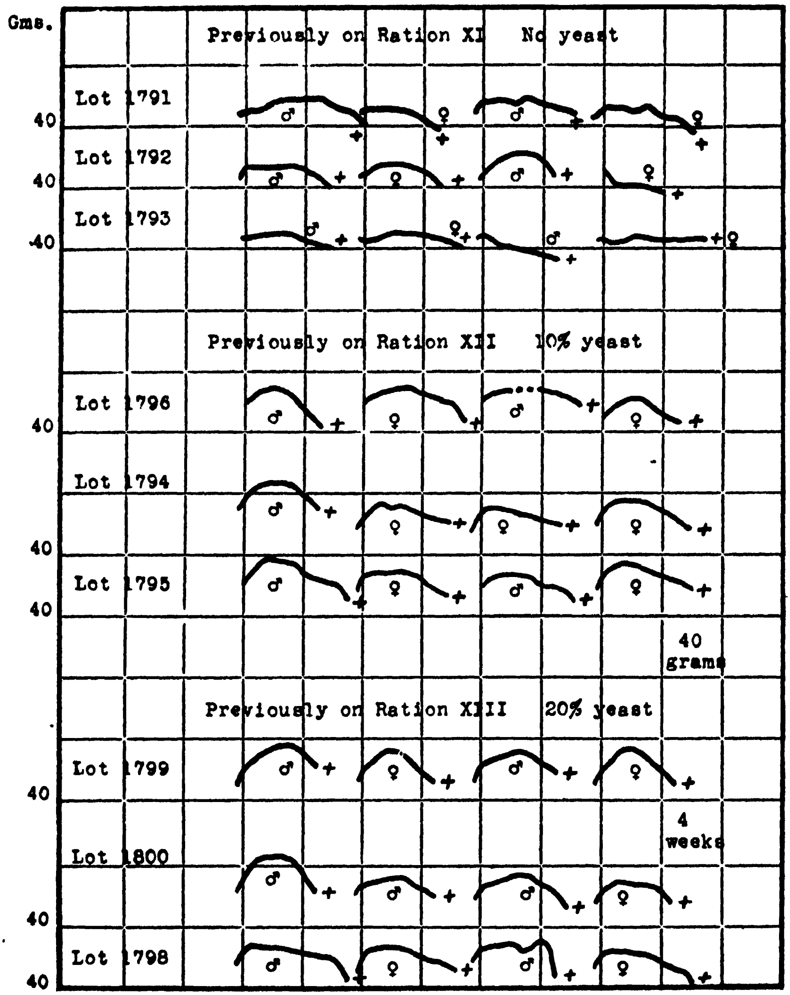

CHART 2. The effect of the previous ration on maintenance in young rats when transferred to a vitamine B-poor ration.

in duration of maintenance of life, gain in weight, or loss in weight before death which could be due to difference of vitamine content of their previous dietary régimes. In fact, the average on

maintenance of life is varied apparently only by the sudden failure of Rat 6041 in Lot 1792, causing one value to be much depressed. The gain in weight, however, appears to be greater with a previously larger vitamine intake as this increased progressively; it, however, is small in proportion to the increase in vitamine content of the ration.

In this latter connection one may well ask the question whether the vitamine intake of the young rats was materially increased by increasing it in the ration fed to the mother. We ask this ques-

TABLE III.

Previous ration.	Yeast.	Lot.	Maintenance of life.	Gain in weight.	Loss in weight from initial weight.	Loss in weight from maximum weight.
	per cent		*days*	*gm.*	*gm.*	*gm.*
XI	None.	1791	55	7	5	12
		1792	37	5*	5	14
		1793	48	6	7†	11
XII	10	1794	49	16	0‡	17
		1795	50	13	6	19
		1796	45	13	12	24
XIII	20	1798	49	16	3§	19
		1799	43	19	0¶	17
		1800	42	15	4‖	17

* One rat lost 7 gm.
† One rat gained 1 gm.
‡ Two rats gained 3 gm.
§ One rat gained 8 gm.
¶ One rat gained 11 gm., one rat gained 5 gm.
‖ Two rats gained 2 gm.

tion because in experiments with cows we have found that milk as ordinarily produced varies very little in its content of vitamine B even when extensive amounts of it are fed with the ration.[1] Probably the same is true of rats, but the fact remains that the rats consume considerable amounts of the mother's ration shortly after opening their eyes, which takes place 15 to 16 days after birth, up to the time that they were put on the vitamine-free ration. In this period, at least, the vitamine intake was increased.

[1] Unpublished data.

The lots started with the rats 32 days old give us more information on this point as they had been weaned for 1 week during which time they had existed and grown actively on their mother's rations. One lot was taken from Ration XII and one from Ration XIII. The data are presented in Chart 3 and Table IV. They show relations not far removed from those obtained with the younger rats (Chart 2 and Table III), maintenance of life not being lengthened, but a somewhat greater gain in weight resulting

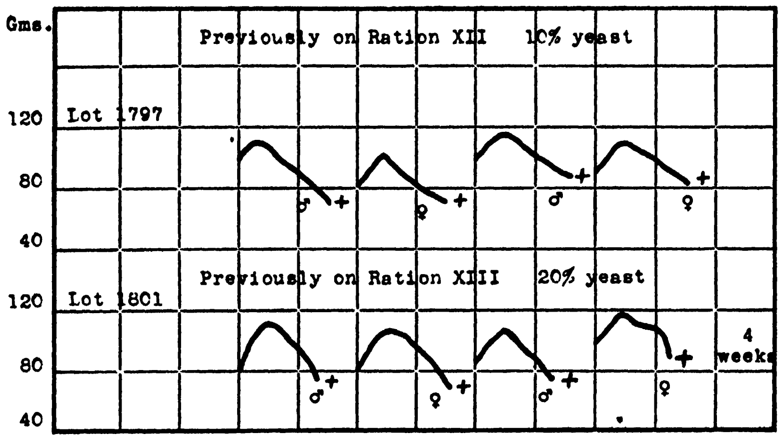

CHART 3. The effect of long continued feeding of a ration high in vitamine B on maintenance in rats when transferred to a vitamine B-poor ration.

TABLE IV

Previous ration.	Yeast.	Lot.	Maintenance of life	Gain in weight	Loss in weight from initial weight	Loss in weight from maximum weight
	per cent		*days*	*gm.*	*gm.*	*gm.*
XII	10	1797	55	14	23	38
XIII	20	1801	42	20	13	34

in those rats which had previously been on the ration of higher vitamine content. This suggests a slight temporary storage of vitamine B, but when considered from the standpoint of intake, at most a very small one, which is in harmony with the fact that we as well as others (3, 4) have observed a prompt failure of growth in rats when put on a vitamine B deficient diet. Here again in comparison with the data shown in Chart 2 and Table III the older

rats lose considerably more in weight before death occurs, though this results after about the same lapse of time as in the younger animals.

CONCLUSIONS.

When young rats of different ages are put on a ration containing little or none of the vitamine B they are able to maintain life for about the same length of time. However, within certain limits, the older and larger the rat the greater the loss in weight before death results. Whether this indicates a need of more vitamine on the part of the larger animal or merely a more disastrous effect of corresponding depletion is not clear.

When young rats reared to 23 to 25 days of age on rations of doubled or quadrupled vitamine B content are subsequently put on a ration containing little or none of the vitamine B they continue to live for the same length of time, but those which previously received generous amounts of vitamine gain in weight more before decline sets in.

When young rats are continued until 32 days of age on high vitamine B rations, maintenance of life is not prolonged, but one fact is emphasized and that is, that after having been on such rations a greater gain in weight results before failure ensues.

All the evidence points to little ability on the part of the rat to store vitamine B.

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COLORIMETRIC DETERMINATION OF IRON AND HEMOGLOBIN IN BLOOD.

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(Received for publication, November 8, 1922.)

Micro methods for the determination of iron in blood based on the comparison of the color of ferric sulfocyanate have been worked out by Berman (1) and more recently by Brown (2). In these methods, the iron in organic combination is set free by the action of acid, the precipitated proteins are filtered off, and the resultant filtrate is then treated with sulfocyanate. Compared with the older procedure of ashing, the methods of Berman and of Brown represent great improvements. But that they are not yet sufficiently simple for general use is shown by the scarcity of blood iron determinations in the literature.

Our experience with these methods has shown that it is essential to remove the proteins completely before treating the solution with sulfocyanate. If some protein passes into the filtrate as it usually does, the color of the unknown will fade rapidly—much more rapidly than that of the standard solution which contains, of course, no protein. The unknown solutions also often show a yellowish tint which renders color reading difficult. In Berman's method, the evaporation of the acetone used for the intensification of the color of ferric sulfocyanate may easily introduce another error into the result.

In the method to be described, the proteins are completely destroyed by the action of concentrated sulfuric acid and sodium or potassium chlorate, and the resulting solution is treated directly with sulfocyanate. The entire procedure is carried out in a single test-tube much in the same fashion as in the determination of total nitrogen by the direct nesslerization method of Folin (3).

The determination of iron in blood by the new method is quite as simple as a determination of the hemoglobin by any of the colorimetric methods. Since the iron in blood is practically equal to the iron in the hemoglobin, we suggest the determination of iron as a convenient substitute for the determination of hemoglobin. The former has the advantage that it is applicable to old as well as to fresh blood and that the standard solution is easily prepared and keeps indefinitely.

The new method is as follows:

Reagents Required.

1. Sodium or Potassium Chlorate.—The chlorate used should be iron-free as shown by a blank test. The high grade C. P. salt is usually good enough. Weigh out the pure chlorate and dissolve in sufficient distilled water to make a 10 per cent solution.

2. Concentrated Sulfuric Acid.—The acid used should be practically iron-free as shown by a blank test.

3. Potassium or Ammonium Sulfocyanate.—Prepare approximately a 3 N solution by dissolving either 146 gm. of C. P. potassium sulfocyanate or 114 gm. of ammonium sulfocyanate in distilled water to make 500 cc. Filter if necessary.

4. Standard Iron Solution.—Weigh out accurately 0.7 gm. of crystallized ferrous ammonium sulfate and dissolve in about 50 cc. of distilled water. Add to the solution 20 cc. of dilute (10 per cent) iron-free sulfuric acid, warm slightly, and then add 0.1 N (approximate) potassium permanganate solution to oxidize the ferrous salt completely. Dilute with distilled water to 1 liter. Each cc. will contain exactly 0.1 mg. of iron for use as a regular standard.

To make stronger or weaker standards for bloods of higher or lower hemoglobin content than normal, dilute the regular standard solution ten times, giving a solution containing 0.01 mg. of iron per cc. As occasion demands, pipette out the number of cc. necessary to give 0.06 to 0.15 mg. of iron.

PROCEDURE.

Measure with an Ostwald pipette 1 cc. of blood into a test-tube, containing exactly 4 cc. of distilled water. Shake thoroughly and

rinse the pipette by drawing the laked blood into it three or four times. Using the same pipette, transfer 1 cc. of this diluted blood to a large Pyrex test-tube, 25 × 200 mm., with graduation at 25 cc. Add 1 cc. of concentrated sulfuric acid free from iron, and a glass bead to prevent bumping. Clamp the test-tube in a burette clamp with adjustable check-nut loosened for ready adjustment. Boil rather vigorously over a micro burner. As soon as the water is boiled off and the test-tube filled with white fumes, cover with a small watch-glass, and continue to boil at the same rate for 3½ minutes. Remove the burner and allow to cool for about 20 seconds. Remove the watch-glass, quickly adjust the test-tube to an angle of about 40° by turning the clamp, and then add, drop by drop, 1 cc. of 10 per cent sodium chlorate solution with a 1 cc. graduated pipette. As the reaction is vigorous, particularly on adding the first few drops, the sodium chlorate should be added with care. Let the drops fall from the pipette onto about the middle of the test-tube. Spattering can also be avoided by the aid of a small funnel with a long stem whose lower end touches about the middle of the test-tube. As soon as the chlorate solution has been added, readjust the test-tube to the vertical position and continue the boiling for another 3 minutes. Again remove the flame, allow to cool for about 20 seconds, turn the test-tube to the former angle, and add 0.3 cc. of chlorate solution as before. To decompose all of the chlorate as well as the last trace of organic matter, adjust the test-tube to the previous position and boil once more for 2 minutes. Cover the test-tube when it is again filled with white fumes. At the end, allow to cool for about a minute, and then add from an ordinary pipette about 5 cc. of distilled water. Avoid spattering by letting the first several drops roll down from the mouth of the test-tube, while the latter is in the inclined position. Add more distilled water to make up about 16 cc. Cool further to room temperature under the tap.

Transfer to another Pyrex test-tube 1 cc. of standard iron solution containing 0.1 mg. of Fe per cc. Add 1 cc. of concentrated sulfuric acid and dilute with distilled water to about 16 cc. Cool to room temperature under the tap. Then add to both the unknown and the standard 5 cc. of 3 N potassium or ammonium sulfocyanate solution and dilute to 25 cc. Insert a clean rubber stopper, mix, and compare in a Duboscq colorimeter.

Calculation.—If the standard is set at 20 mm., then 20 divided by the reading of the unknown and multiplied by 50 gives the number of mg. of Fe in 100 cc. of blood. To obtain the percentage of hemoglobin, divide this number by 3.35, since hemoglobin contains 0.335 per cent of iron (4, 5).

$$\frac{20}{R} \times 50 = \text{mg. of Fe per 100 cc. of blood.}$$

$$\frac{20 \times 50}{R \times 3.35} = \text{percentage of hemoglobin in blood.}$$

Accuracy.—In order to ascertain the accuracy of the new method, a comparison has been made of our results with those obtained by Wolter's volumetric method (6) applied to the ash of 5 cc. of blood. The close agreement is shown in Table I. The accuracy of the method is also shown by the analysis of a sample of pure methemoglobin (Table II). The applicability of the method to hemoglobin determinations is shown in Table III where the percentages of hemoglobin calculated from the iron contents are compared with those obtained by Palmer's carbon monoxide method (7).

TABLE I.

Comparative Determination of Iron in Blood by Wolter's Volumetric Method and by the New Colorimetric Method.

Source.	Volumetric method.	Colorimetric method.
	mg. per 100 cc.	*mg. per 100 cc.*
Dog 1	36.4	36.6
" 2	41.3	41.0
Chicken 1	37.4	37.3
" 2	33.7	33.8
Sheep 1	43.3	43.3
" 2	50.6	50.8
" 3	46.8	46.4

TABLE II.

Determination of Iron in Solutions of Pure Methemoglobin of Dog.

Solution.	Concentration.	Fe found.	Fe calculated.
	per cent	*mg. per 100 cc.*	*mg. per 100 cc.*
1	2.5	8.39	8.38
2	3	10.05	10.05
3	3.5	11.76	11.74
4	4	14.45	14.40

TABLE III.

Comparison of Hemoglobin Determinations by Palmer's Method and by the Iron Method.

Sample.*	Fe method.	CO method.
1	14.3	14.5
2	15.1	15.0
3	14.5	14.9
4	15.0	15.2
5	17.3	17.7
6	15.2	15.2
7	15.4	15.7
8	15.9	16.1

* The blood left from Wassermann's test.

SUMMARY.

A simple and rapid colorimetric method is described for the determination of iron in blood.

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THE USE OF PERSULFATE IN THE ESTIMATION OF NITROGEN BY THE ARNOLD-GUNNING MODIFICATION OF KJELDAHL'S METHOD.

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It is the purpose of this paper to show that the period of digestion in the Arnold-Gunning (1, 2) modification of the Kjeldahl method can be shortened under favorable conditions to one-third with the aid of potassium persulfate. Huguet (3) added a concentrated solution of sodium persulfate to urine before digesting with hot sulfuric acid for the gasometric estimation of urinary nitrogen. In Pittarelli's (4) simplified Kjeldahl method for clinical purposes, crystals of potassium persulfate are dropped into the boiling digestion mixture. Our experience with the regular Kjeldahl method in this connection has, however, shown that the most favorable condition for the persulfate to give full effect is at the latter stage of the digestion and in the presence of moisture. Added at the beginning of the process, the persulfate serves not much better than the plain sulfate recommended by Gunning. Added at the end without the assistance of moisture, the persulfate would decompose too rapidly and the oxidation become less effective. Moreover, the greater part of the decomposition would have taken place before the salt reached the boiling digestive mixture if the flask had not been allowed to cool a while first.

Discounting the time consumed in boiling off the water at the beginning and at the end of the new process, the total intensive heating seldom exceeds 40 minutes and is in most cases much less than 30 minutes. Therefore, the chances of losing, a common occurrence in the old method of prolonged heating, a portion of the preformed ammonium sulfate are greatly diminished. With the digestive mixture cooled for 10 minutes before the introduc-

tion of moisture, the oxidation by the persulfate takes place at a comparatively low temperature and the danger of explosion is thus avoided entirely. Furthermore, no loss of nitrogen through such oxidation has been encountered.

The new modification is as follows:

PROCEDURE.

To a clean 800 cc. Kjeldahl flask transfer 5 cc. of urine, diluted (1:5) blood, or milk, or 0.5 gm. of dry protein. Wash down with as little water as possible any material adhering to the neck of the flask. Add 2 cc. of 5 per cent copper sulfate, 5 gm. of pure potassium sulfate, 20 cc. of concentrated nitrogen-free sulfuric acid, and a few clean quartz pebbles. Heat on the digestion shelf, first gently until the vigorous frothing ceases, and then strongly so that the liquid boils vigorously. When the acid mixture becomes amber-colored (this point is usually reached in 20 to 30 minutes, less than 10 minutes with urine), turn off the flame and allow to cool for 10 minutes. At the end of this period, remove the flask from the shelf, holding the neck with a towel. While holding the flask with its mouth pointing away from the face, gradually let about 3 cc. of distilled water, from a pipette or a dropper, flow down the side of the flask to the hot acid mixture, at the same time rotating gently. As soon as this is done, set the flask upright and dump 10 gm. (3 gm. in the case of urine) of potassium persulfate into it, taking care that as little powder as possible adheres to the side of the neck. Rotate the flask to have a thorough mixing and reheat immediately. Continue the heating until the acid mixture becomes green. This usually takes about 15 minutes. Cool for 10 to 15 minutes and while agitating the flask under the tap gradually add about 300 cc. of distilled water. Add ½ spoonful of talcum powder to prevent bumping. Now conduct the distillation of ammonia in the usual manner, using at least 50 cc. of 0.1 N H_2SO_4 in the case of urine, 30 cc. in the case of blood and of milk, 25 cc. of 0.5 N H_2SO_4 in the case of dry protein, to absorb the ammonia distillate.

Accuracy.—In order to ascertain the accuracy of the new modification, comparative study has been made with the old modification of Arnold-Gunning. Their close agreement is shown in Table I.

Rapidity.—The most important feature of the new modification is its superiority in speed. To show this, comparison has been made with the old method in analyzing a sample of milk. In the

TABLE I.

Comparative Determinations of Total Nitrogen by the Old and by the New Modifications of Kjeldahl's Method.

Source.	N determination.	
	New modification.	Old modification.
	mg. per cc.	*mg. per c.c.*
Urine 1	10.76	10.69
" 2	12.00	11.86
" 3	11.20	11.15
Blood 1	27.32	27.18
" 2	33.80	33.69
" 3	36.90	36.73
Milk 1	4.76	4.72
" 2	5.92	5.89
" 3	5.60	5.53
" 4	6.00	5.92
	mg. per gm.	*mg. per gm.*
Egg albumin 1	128.03	127.80
" " 2	128.65	127.78
Gelatin 1	159.60	158.97
" 2	158.99	158.80

TABLE II.

Progress of Digestion by the Old Method.

Time in hrs.	½	1	1½	2	2½	3	3½
N obtained, *mg.*	14.8	21.2	21.8	22.3	23.0	23.3	23.3
Per cent of total	63.7	91.2	93.7	95.5	98.8	100	100

new method, the digestion of the milk is completed in 40 minutes of actual heating; whereas in the old method it takes about 2½ hours. The comparative velocities of these methods are shown in Table II and Fig. 1.

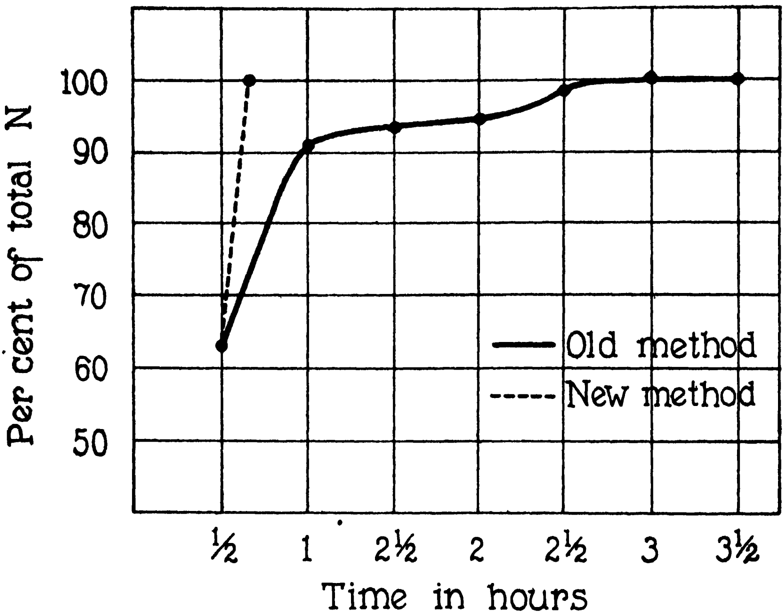

FIG. 1. The velocity curves of digestion by the old and by the new method.

SUMMARY.

The use of persulfate is recommended in the determination of total nitrogen by the Arnold-Gunning modification of Kjeldahl's method.

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THE USE OF PERSULFATE IN THE ESTIMATION OF NITROGEN BY FOLIN'S DIRECT NESSLERIZATION METHOD.

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(Received for publication, November 22, 1922.)

We have in the foregoing communication shown that potassium persulfate can be used to accelerate considerably Kjeldahl digestion. In our endeavor to eliminate entirely the interference of silica, as frequently occurs in Folin's direct nesslerization method,[1] we have tested and found potassium persulfate to be also of great service. Using sulfuric acid alone for the preliminary digestion and later adding potassium persulfate solution as oxidizing agent, even difficultly digestible substances, such as milk, have not been found to give any trouble. The resultant solution always remains clear.

As different substances contain a varying amount of organic matter, slightly different procedures have been worked out for urine, blood, and milk, respectively. One of these procedures suitably chosen, will be found applicable to other solutions containing organic nitrogen.

The new method is as follows:

Reagents Required.

Diluted Sulfuric Acid.—Gradually pour 50 cc. of N-free concentrated sulfuric acid into a 300 cc. Erlenmeyer flask containing 50 cc. of distilled water, keeping it cool under the tap. Keep the diluted acid in a glass stoppered bottle. Use 1 cc. for each digestion. The acid used should be nitrogen-free as shown by a blank test.

[1] Folin, O., Laboratory manual of biological chemistry, Boston, 2nd edition, 1919, 181.

Saturated Potassium Persulfate.—The persulfate used should be nitrogen-free as shown by a blank test. Shake up in a small glass stoppered bottle about 7 gm. of potassium persulfate with 100 cc. of distilled water. The undissolved part settles on the bottom and keeps the solution saturated even if there be slight decomposition.

Nessler's Solution.—The solution is prepared according to Folin's direction[2] as follows:

"Transfer 150 gm. of potassium iodide and 110 gm. of iodine to a 500 cc. Florence flask; add 100 cc. of water and an excess of metallic mercury, 140 to 150 gm. Shake the flask continuously and vigorously for 7 to 15 minutes or until the dissolved iodine has nearly all disappeared. The solution becomes quite hot. When the red iodine solution has begun to become visibly pale, though still red, cool in running water and continue the shaking until the reddish color of iodine has been replaced by the greenish color of the double iodide. Now separate the solution from the surplus mercury by decantation and wash the latter with liberal quantities of distilled water. Dilute the solution and mixed washings to a total volume of two liters.

"Introduce into a large bottle 3700 cc. of 10 per cent sodic hydrate solution, add 750 cc. of the double iodide solution, and 450 cc. of distilled water. This gives 5 liters of Nessler's solution."

Standard Ammonium Sulfate Solution.—Prepare a stock solution by dissolving 4.716 gm. of specially purified ammonium sulfate in distilled water and make the volume up to 1 liter. For use, dilute 100 cc. of the stock solution to 1,000 cc. This makes a standard solution, each cc. of which contains 0.1 mg. of nitrogen.

Procedure for Urine.

Regarding the average amount of total nitrogen in urine as being about 25 times as great as the "ammonia" nitrogen, dilute the urine so that each cc. will contain 0.2 to 0.3 mg. of nitrogen. 2 cc. of urine diluted to 100 cc. usually gives the right nitrogen content. Transfer with an Ostwald pipette 1 cc. of the diluted urine to a large Pyrex test-tube, 200 $\times$ 25 mm., marked at 35 and at 50 cc. Add 1 cc. of the diluted sulfuric acid (1:1) and a quartz pebble. Hold the test-tube with a test-tube holder and boil vigorously with constant shaking over a micro burner until a greater part of the water is expelled. Then clamp the test-tube

[2] Folin,[1] p. 203.

in a burette clamp and continue the boiling until white fumes begin to fill the tube. Should the boiling come to a standstill, tap the test-tube to avoid bumping. As soon as the test-tube is nearly full of fumes, cover with a small watch-glass and reduce the flame so that the acid mixture boils gently. Continue the gentle boiling for 2 minutes, counting from the time the test-tube becomes filled with fumes. Remove the burner and allow to cool for 1 minute. Take off the watch-glass and add 2 drops of saturated potassium persulfate with a fine pipette or a dropper. Replace the burner and continue the boiling until the digestion mixture becomes colorless. Stop the boiling about 15 seconds after the reappearance of the white fumes, the test-tube being covered with a watch-glass during this period. Allow to cool for 70 to 90 seconds. Then add 20 to 25 cc. of distilled water. Cool to room temperature under the tap. Dilute with distilled water to the 35 cc. mark.

To another similar Pyrex test-tube measure exactly 2 cc. of standard ammonium sulfate solution containing 0.1 mg. of nitrogen per cc. Add 1 cc. of the diluted sulfuric acid and dilute to the 35 cc. mark with distilled water. Now add to both the unknown and the standard 15 cc. of Nessler's solution, using an ordinary pipette of large aperture; let the Nessler's solution fall directly into the acid solution so that it is thoroughly mixed thereby. Insert a clean rubber stopper, mix, and compare in a Duboscq colorimeter.

Calculation.—If the standard is set at 20 mm. in the color comparison, 20 divided by the reading and multiplied by 0.2 (or 0.3 if 3 cc. of standard solution be used) and 50 (or the figure according to the dilution of the urine) gives the number of mg. of nitrogen in 1 cc. of urine. This number multiplied by the number of cc. of urine per 24 hours will give the total nitrogen in the 24 hour urine.

Procedure for Blood.

Dilute the blood 150 times and determine the total nitrogen in 1 cc. as in urine with the modification that heating should be continued after the test-tube becomes filled with fumes, 3 minutes instead of 2; and that the amount of saturated potassium persulfate solution used should be 0.5 cc. instead of 2 drops.

Procedure for Milk.

Dilute the milk 20 times and determine the total nitrogen in 1 cc. as in urine with the modification that the heating should be longer and more intense. After the test-tube becomes filled with fumes, heat 4 minutes instead of 2. Also the amount of saturated potassium persulfate solution used should be 1 cc. Greater care should be taken in adding the persulfate solution. After that, continue the heating until the digestive mixture becomes colorless. This requires about 2 minutes after the reappearance of white fumes.

Accuracy.—In order to ascertain the accuracy of the new method, we have compared our results with those obtained by the Kjeldahl method. In the case of urine, comparison has also been made with Folin's method. The close agreement of the comparative determinations of total nitrogen in urine, blood, and milk is shown in Tables I to III.

TABLE I.

Comparative Determinations of Total Nitrogen in Urine by Kjeldahl's Method, by Folin's Method, and by the New Method.

Sample No.	New method.	Folin's method.	Kjeldahl's method.
	mg. per cc.	*mg. per cc.*	*mg. per cc.*
1	10.8	10.7	10.9
2	12.2	12.3	12.5
3	14.4	14.4	14.3
4	12.9	13.0	12.8
5	12.6	12.7	12.4

TABLE II.

Comparative Determinations of Total Nitrogen in Human Blood by Kjeldahl's Method and by the New Method.

Sample No.	New method.	Kjeldahl's method.
	mg. per cc.	*mg. per cc.*
1	29.4	29.5
2	33.0	32.8
3	26.8	26.7
4	28.2	28.2
5	32.4	32.7
6	26.3	26.4

TABLE III.

Comparative Determinations of Total Nitrogen in Milk by Kjeldahl's Method and by the New Method.

Sample No.	New method	Kjeldahl's method
	mg per cc.	*mg. per cc.*
1	4.15	4.20
2	3.88	3.85
3	5.80	5.74
4	4.76	4.76
5	2.96	2.93

SUMMARY.

The use of persulfate is recommended in the determinations of total nitrogen in urine, blood, and milk by Folin's direct nesslerization method.

THE ACTION OF DIAZOMETHANE ON XANTHOSINE.

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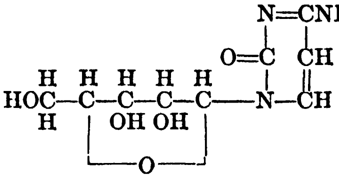
(Received for publication, January 23, 1923.)

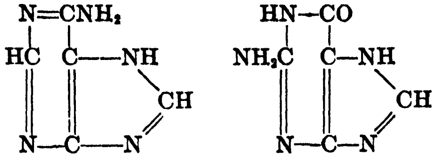
In nucleosides the position of ribose on the purine and pyrimidine bases, respectively, has not as yet been adequately established.

As regards the pyrimidine nucleoside, position 3 may be accepted as the place of union. It is probable that the pentose radicle is attached to a nitrogen and not to a carbon atom of the base. The velocity of hydrolysis of the hydrogenated pyrimidine nucleoside excludes the possibility of a carbon to carbon union, since a substance of that structure would display unusual resistance towards hydrolytic agents. Since in cytidine the nitrogen atom in position 4 is present in a primary amino group; therefore, the nitrogen atom in position 1 is not reactive, hence on theoretical considerations the following structure may be assigned to this nucleoside:

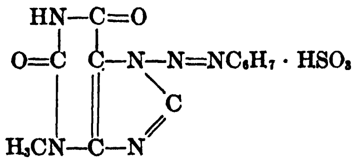


Again, since cytidine is readily transformed into uridine, the latter undoubtedly possesses a structure analogous to that of the former. Experimentally, this theory has not as yet been proved, but work on this problem is now in progress.

In purine nucleosides the problem is more complex, since the number of nitrogen atoms in the purine ring is double that in the pyrimidine ring. The structures of adenine and guanosine are as follows:



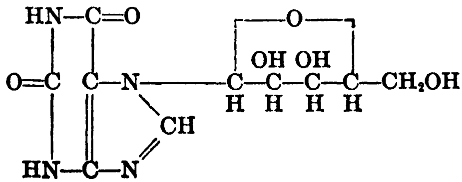
Since the amino group in neither adenosine nor guanosine is substituted, position 6 is excluded from consideration for adenine and position 2 for guanine. The nitrogen atoms in position 7 or 9 in adenine and the nitrogen atoms 1 and 7 or 1 and 9 in guanine permit of substitution. Since adenosine is readily converted into inosin, it is evident that the mode of union of base and pentose is the same for the two nucleosides. At a time when the details of the structure of nucleic acids was little known Burian suggested that nitrogen atom 7 may be the one connecting the base with the rest of the nucleic acid molecule. The reason for his conclusion was the fact that purines substituted in position 7 as well as nucleic acids did not form a dye with diazobenzenesulfonic acid, whereas purines, non-substituted in position 7 formed a dye to which Burian assigned the structure of a diazoamino compound as follows:



Pauly and, later, Hans Fischer pointed out that the properties of the substances described by Burian suggested the structure of an azo dye rather than that of a diazoamino compound. Fischer advanced experimental evidence in support of his view. He has also shown that purines substituted in position 8 failed to couple with diazo compounds. The choice of the position of the union between the base and the rest of the molecule was limited by Fischer to 7 and 8. It was pointed out above that position 8 may be ruled out on theoretical grounds. Fischer's findings now suggest that the pentose and base are united through position 7 of the base. However, the experience of Fischer has demonstrated that the reactivity of the individual hydrogen atoms of the base is influenced by substitutions in the other atoms and, therefore,

more direct evidence as to the place of union between base and pentose is greatly desired.

Since the discovery of the nucleosides many attempts have been made in this laboratory to methylate the nucleosides in the hope that by hydrolysis of the methylated products the place of union with the base would be detected by a direct method. After many unsuccessful trials conditions were finally found which led to the formation of dimethyl xanthosine. This substance on hydrolysis yielded a dimethyl xanthine, melting sharply at 265°C., which therefore is 1-3-dimethyl xanthine (theophylline). Thus it is evident that in xanthosine the ribose radicle is attached to the nitrogen atom in position 7 of the base. Xanthosine may be regarded as 7-ribose xanthine. The structure of xanthosine is then fully expressed as follows:



It was curious to note that the action of diazomethane did not end with the formation of dimethyl xanthosine. Besides the methylated nucleoside trimethyl xanthine (caffeine) and, in one experiment in which the methylation was continued longer than usual, tetramethyl uric acid were isolated from the reaction products. The hydrolytic and oxidizing actions of diazomethane and their mechanisms deserve special investigation.

It may be mentioned here that on methylation of guanosine a crystalline monomethyl derivative was obtained, and on hydrolysis the crude reaction product yielded 1-methyl guanine. So far there has not been sufficient material on hand to carry out the hydrolysis on crystalline methyl guanosine. Because of this the publication of the details of the work on guanosine will be postponed.

EXPERIMENTAL.

The preparation of xanthosine was carried out according to the directions of Levene and Jacobs.[1] Only a slight modification

[1] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1910, xliii, 3163.

was introduced concerning the use of sodium nitrite and glacial acetic acid. Portions of 20.0 gm. of guanosine were dissolved in 200 cc. of hot water containing 50.0 gm. of sodium nitrite. The solution was stirred while cooling in order to prevent it from turning into a jelly. When the solution is cooled to room temperature (between 25–30°C.) 100 cc. of glacial acetic acid are added gradually. It takes 1 to 1.5 hours for the guanosine to dissolve completely. An equal volume of ice-cold water is then added, and on scratching, xanthosine began to crystallize immediately. After 24 hours standing at 0°C. the crystallization was complete. The yield of guanosine was about 12 to 13 gm. For methylation the material was recrystallized twice.

Methylation of Xanthosine.

A closed vessel containing 10 gm. of dry xanthosine and 150 cc. of dry methyl alcohol was placed in a shaking apparatus. Freshly distilled diazomethane in portions of about 1.0 gm. was added in rapid succession as soon as the solution decolorized. The first portions generally decolorized immediately. The later portions kept their color for some time, and the final portion for about 3 hours. At the end of that time the greatest part of the xanthosine was dissolved. As a rule about 4.0 gm. of xanthosine remained undissolved. The filtrate was then concentrated under diminished pressure practically to dryness, absolute alcohol was added to the flask, and the alcohol distilled off under diminished pressure. This operation was repeated twice. The residue was then taken up in boiling alcohol (98.5 per cent) and poured into about 4 liters of dry (over sodium) ether. An amorphous precipitate and filtrate are obtained which will be referred to, respectively, as the methyl xanthosine and the caffeine fractions.

Caffeine Fraction.

The ethereal solution was concentrated to nearly dryness. The residue generally crystallized on short standing. However, for purification it was dissolved in a little alcohol and poured into a large excess of dry (over sodium) ether. A small amorphous precipitate was removed by filtration and the filtrate was concentrated to dryness. The residue was taken up in very little boiling alcohol and allowed to cool. The substance crystallized

in long colorless needles. The crystallization was completed in about 24 hours. For purification the substance was recrystallized from absolute alcohol. The substance melted at 237°C. and analyzed as follows:

0.1012 gm. substance:	0.1822 gm. CO_2 and 0.0478 gm. H_2O.		
0.0984 " "	required (Kjeldahl) 20.35 cc. 0.1 N acid.		
$C_8H_{12}N_4O_2$.	Calculated.	C 49.48, H 5.15, N 28.86.	
	Found.	" 49.09, " 5.28, " 28.94.	

In one experiment the addition of diazomethane was continued until the last portion retained its color for nearly 8 hours. The caffeine fraction crystallized in the form of heavy prisms and intermingled with it were a few needle-shaped crystals. On recrystallization the needles disappeared. The substance then analyzed for tetramethyl uric acid as follows:

0.1078 gm. substance:	0.1906 gm. CO_2 and 0.0532 gm. H_2O.	
0.0989 " "	required (Kjeldahl) 18.05 cc. 0.1 N acid.	
$C_9H_{12}N_4O_3$.	Calculated.	C 48.23, H 5.36, N 25.01.
	Found.	" 48.03, " 5.52, " 25.55.

Dimethyl Xanthosine Fraction.

The crude material was taken up in boiling absolute alcohol, a small part remaining insoluble. The mixture was cooled to about 0°C., the insoluble part removed by filtration, and the filtrate poured into about 4 liters of dry (over sodium) ether. The crude amorphous precipitate had an elementary composition which agreed fairly satisfactorily with that required for dimethyl xanthosine. After repeating the above treatment in alcohol and ether twice, the elementary composition of the substance remained unchanged. For analysis the substance was dried to constant weight under diminished pressure at the temperature of the vapors of boiling carbon tetrachloride.

Analysis of the original material.
0.1026 gm. substance: 0.1720 gm. CO_2 and 0.0500 gm. H_2O.
0.0948 " " required (Kjeldahl) 12 cc. 0.1 N acid.

Analysis of the twice reprecipitated material.
0.1020 gm. substance: 0.1708 gm. CO_2 and 0.0500 gm. H_2O.
0.0940 " " required (Kjeldahl) 11.70 cc. 0.1 N acid.

$C_{12}H_{16}O_6N_4$.	Calculated.	C 46.12, H 5.16, N 17.95.
	Found, original sample.	" 45.71, " 5.45, " 17.72.
	" purified "	" 45.66, " 5.48, " 17.42.

The rotation of the substance was

$$[\alpha]_{D}^{20} = \frac{-0.28° \times 100}{1 \times 1} = -28°$$

Hydrolysis of Dimethyl Xanthosine.

Whereas the unsubstituted nucleosides on boiling with dilute acids such as 0.2 N sulfuric or hydrochloric for 1 to 2 hours are hydrolyzed without melanin formation, dimethyl xanthosine forms considerable melanin on boiling with such acids for only 30 minutes. The formation of melanin is accompanied with a disappearance of the base. In the experiments in which the hydrolysis was effected by heating for 1 hour in an autoclave at 105°C., inside temperature, the product of hydrolysis was a deeply colored solution in which solid melanin was suspended. The yield of the base in such experiments was small. Therefore, it was found advantageous to interrupt the hydrolysis at a phase when only part of the methylated nucleoside was hydrolyzed, but before the products of hydrolysis began to transform into melanin. For this reason the hydrolysis was interrupted as soon as the solution began to darken. The procedure finally adopted was as follows: 10.0 gm. of the dimethyl xanthosine were dissolved in 500 cc. of 2 per cent sulfuric acid and heated with reflux over a free flame for 30 minutes. The operation was then interrupted, the sulfuric acid was removed by means of lead carbonate, the dissolved lead by means of hydrogen sulfide, the filtrate was concentrated to nearly dryness then taken up in 98.5 per cent alcohol, and the solution again concentrated to dryness under diminished pressure. This operation was repeated twice. Finally, the residue was dissolved in about 300 cc. of 98.5 per cent alcohol and the solution poured into 4 liters of dry (over sodium) ether. A precipitate formed which consisted in the main of unchanged material. It weighed about 6.0 gm. The filtrate was concentrated to dryness and a crystalline precipitate was obtained. This was twice recrystallized from a mixture of ethyl and methyl alcohol and finally from water. The yield of the purified material was very small. The substance crystallized in colorless prismatic platelets. The melting point was sharp at 265°C. (corrected). The substance analyzed as follows:

0.1000 gm. substance required (Kjeldahl) 32.15 cc. 0.1 N acid.

$C_7H_8N_4O_2$.	Calculated.	N 31.11.
	Found.	" 31.01.

Thus the substance is 1-3-dimethyl xanthine.

THE NUTRITIVE PROPERTIES OF MILK WITH SPECIAL REFERENCE TO REPRODUCTION IN THE ALBINO RAT. II.

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To explain the inability of rats to reproduce on a milk diet (1) it was suggested that milk might be lacking in substances necessary for successful adolescent growth and reproduction or that it might contain substances inhibitory of growth in the adolescent and mature growth periods. The latter hypothesis was made necessary by the findings of Osborne and Mendel (2) who reported reproduction in the case of two animals on a ration of dry milk 60 per cent, lard 28 per cent, and starch 12 per cent and also by our own partial success with rats on a ration containing 55 per cent whole milk powder.

Rations with Varying Proportions of Milk.

In order to determine whether the dilution of an inhibitory factor in milk could turn reproductive failure into success a number of animals were placed on a series of rations containing 80, 70, 60, and 50 per cent of milk powder, 10 per cent lard, 0.8 to 2 per cent salt mixture (McCollum's No. 185), and starch. Some were also placed on the dry milk ration of Osborne and Mendel, as well as on this ration with an additional 2 per cent of the salt mixture. A few were also placed on whole milk powder with 0.2 per cent iron citrate. The animals were 25 to 35 days of age when placed on these rations and weighed from 30 to 40 gm. Matings were always with animals from other litters. Of the 59 animals 5 died from various causes and 54 were followed through adolescence and the usual reproduction period.

The average rate of growth of these animals is indicated in Table I and in Charts 1 and 2 and it appears that while the growth

TABLE I.

Average Weight of Animals on Dry Milk Rations.

Rations.		Average weight of females.				Average weight of males.		
		75 days.	125 days.	175 days.		75 days.	125 days.	175 days.
		gm.	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*
Milk powder 50, starch 38, lard 10, salts 2	(4)*	117	159	174	(3)*	137	211	234
" " 60, " 28.4, " 10, " 1.6	(5)	119	158	172	(5)	143	209	240
" " 70, " 18.8, " 10, " 1.2	(5)	128	167	191	(5)	141	213	237
" " 80, " 9.2, " 10, " 0.8	(6)	113	140	160	(1)	154	208	
" " 100, iron citrate 0.2 per cent	(2)	122	144		(2)	133	154	
" " 60, starch 12, lard 28†	(4)	108	149	148	(3)	134	210	
" ". 60, " 12, " 26, salts 2	(6)	100	140	157	(3)	126	189	219
Donaldson's standard		106	170	194		122	198	245

* The figure in parenthesis indicates the number of animals in the average.

† Osborne and Mendel's ration.

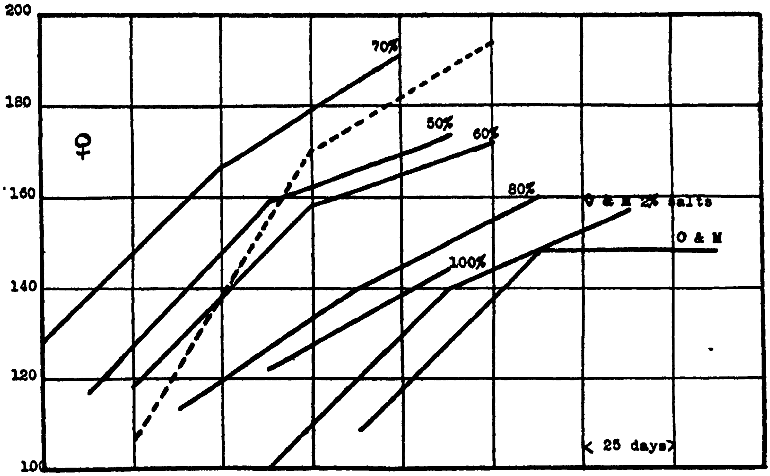

CHART 1.

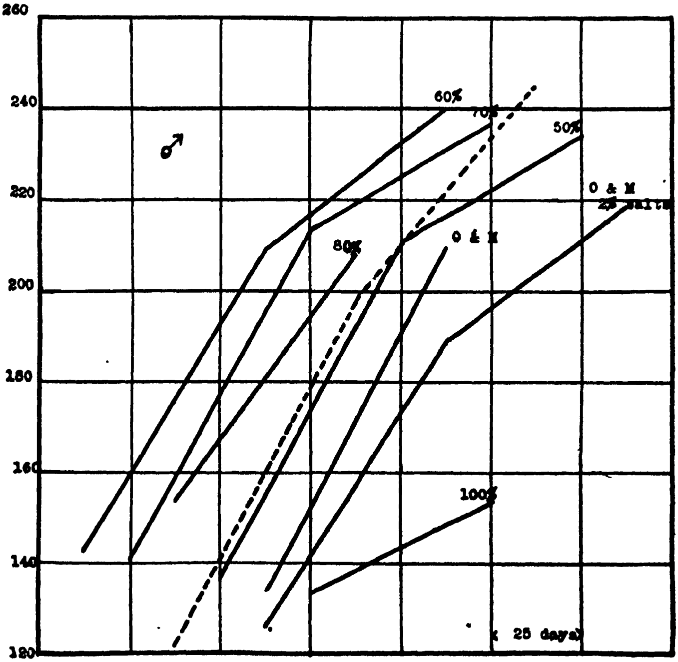

CHART 2.

CHARTS 1 and 2. Average weights of animals at 75, 125, and 175 days of age on rations containing different percentages of whole milk powder. For the composition of the rations see Table I.

rate up to 75 days of age was more rapid than normal on practically all of these rations, it began to decline thereafter. At 125 days males were still up to standard with the exception of those on dry milk powder and iron citrate while females were all below the expected figure. At 175 days both sexes were below standard, males about 5 per cent and females 14 per cent. These data thus confirm the earlier observations as to the greater susceptibility of females to the stunting effect of an all milk diet.

The number of animals on each ration is too small to establish definite superiority as between the different milk percentages. There is, however, a fairly sharp demarcation in the female animals (where it might be expected) between the lower and higher percentages of milk. 80 and 100 per cent are distinctly inferior to 50, 60, and 70. The failure of the 60 per cent milk powder with 26 or 28 per cent lard, may be attributed to the smaller protein and vitamine intake consequent upon the high calorie diet. The figures of the male animals are unfortunately incomplete because some of the animals were put on other rations or were killed for examination before the age of 175 days. The figures at 125 days, however, indicate that the lower percentages of milk are equally as good as, if not better than, the higher.

If the rations with lower percentages of milk seemed to promote better growth than an all milk diet this superiority was not revealed in reproduction. Reproduction was not successful on any of these rations.[1] Among the thirty-two females there were six pregnancies and the young were seen in only two instances.

[1] From personal communications with Dr. H. C. Sherman at Columbia University it appears that he has had success in rearing fertile rats upon an all milk ration at least to the second generation. In this laboratory the first attempts (1) to secure growth and reproduction on milk alone were carried out on animals which were the first generation of Wistar Institute stock. Those attempts were not successful. Results similar to our own were reported by Winfield (Winfield, G., Some investigations bearing on the nutritive value of dried milk, *Gt. Britain Local Gov. Bd., n. s. No. 116; Food Rep., No. 24,* 1920, 148). The stock from which the experimental animals described in this paper were derived were descendants of the original colony, without inbreeding and without any new strains. Since the colony is small, extensive selection for vigor could not be practised and it is entirely possible, therefore, that our animals were not as vigorous as those in the Columbia University colony. Parasites are not responsible for any loss in vigor as the colony is free from them.

Evidence for the others was found in the characteristic drop in weight, not over 15 to 20 gm., or in the stains in the filter paper bedding, or in the presence of placental scars, post mortem. Of the six animals in question two were on 50 per cent milk, one on 60 per cent, and one on 80 per cent; the two others had been on the Osborne-Mendel mixture (milk powder 60, lard 28, and starch 12 per cent); during gestation one was transferred to stock ration, the other was given dry yeast *ad libitum* but their litters were dead when found or died in a day or two.

The inadequacy of milk as the sole source of protein and vitamines seemed to produce a permanent incapacity; when animals at the age of 150 to 175 days were transferred from these rations to the stock rat food they did not develop reproductive ability within the time of observation, 30 to 147 days. Others were transferred variously to milk rations containing added protein-free milk, cod liver oil, yeast, or a salt mixture containing iodide, but with no effect on reproduction except in one case in which a female after 82 days on a ration with 5 per cent of yeast, and at the age of 203 days gave birth to a litter of nine. Six of these lived and made inferior growth on the same ration. They were sired by a stock male. Of these and other yeast-fed animals mention will be made later.[2]

To determine whether male and female rats are equally affected by the inadequacies of a milk ration stock female or male rats were mated with experimental animals for periods of 1 to 2 weeks and then returned to stock ration. Of the thirty-two females eighteen were thus tested with stock males, twelve once, the others two and three times. No pregnancies resulted except the one already mentioned (on 5 per cent yeast addition). Tests on eight males with stock females were negative in five cases; in the remaining three, pregnancies resulted and the young lived from 1 to 6 weeks. From these results it would appear that impairment of reproduc-

[2] In view of the explanation given by Daniels and Loughlin (Daniels, A. L., and Loughlin, R., *J. Biol. Chem.*, 1920, xliv, 381), for a deficiency in heat-treated milk, three pairs of animals were placed on a ration of milk powder 89.5, lard 10, and $Ca_3(PO_4)_2$ 0.5 per cent. One of these animals grew to a weight of 160 gm., the others all remained below 125 gm. Their food consumption was low. It is doubtful that dry milk suffers from the deficiency in question in our experiments.

tive capacity like that of growth, is more marked in females than in males, also that a ration whose entire protein and vitamine supply comes from milk is not completely adequate for either sex.

Milk Rations Containing Yeast.

Information on the effect of lack of vitamine B upon reproductive efficiency has lately been amplified by the results of Dutcher and Wilkins (3) on cockerels and Novaro (4) on pigeons and by the recent development of the technique of Evans and Bishop (5) for observing the œstrous cycle in rats. A shortage of vitamine B did not seem a valid explanation of the failure of our rats on milk rations because the daily intake of dry milk even on a 50 per cent ration is far above the limiting requirement (6, 7) unless the process of procreation makes more than a threefold demand above that of ordinary growth. The addition of wheat embryo had not proved effective in the limited trials which it was given and this substance is again being tested.[3] The addition of yeast, while probably not so good a source of vitamine B (8), had proved more effective and consequently a number of animals, four males and nine females, were started on rations containing 60 per cent of milk powder and 5 per cent of dry yeast. This yeast contained no starch; it was supplied in moist condition by the Fleischmann Company and was dried in a current of warm air (25–30°C.). At ages of 75 and 125 days the average weight of the males was 148 and 212 gm., respectively; that of the females 117 and 157 gm., respectively; figures which are almost identical with those obtained on the 60 per cent milk ration without yeast. However, all but one of the females had litters sired by males on the same ration; four had one litter each, two had two litters each, and two had three litters each. None of the young lived; several grew to have hair but usually they died within the first 2 days or were not seen at all. The litters seen averaged six to seven young.

The striking contrast between animals on milk rations with and without yeast is difficult to explain on any other grounds

[3] Modifying the dietary of a grown animal, while an important and necessary procedure in some lines of work, has in this connection not been as satisfactory as starting a young animal on the modified ration; until the various factors are better understood more consistent results may be expected by starting at the same base line.

than the yeast addition. The females came from four different litters, the males represented two others, and litter mates on milk rations without yeast were entirely infertile. Among the causes for the loss of the young was lack of milk on the part of the mothers; the white stomach which a nursing rat shows through the abdominal wall was absent. Nor did the mothers display any maternal instinct. No nests were made of the bedding supplied (paper towels) and the young were left scattered about the cage. It is interesting to speculate upon a possible connection between the two facts.

Variations in the Weight of Gonads.

The gonads of most of the milk-fed animals were weighed and their weight was compared with the expected normal according to Donaldson's standard. Of fourteen males examined five were above and nine below normal. The average overweight was 12 per cent while the average underweight was 39 per cent. Those whose testes were of normal weight or above averaged 149 days of age. Those with underweight testes averaged 203 days of age. Of twenty-eight females only eight were found to be above normal; twenty were below normal. The average overweight was 24 per cent, the average underweight was 39 per cent. Both groups averaged 213 days of age. The number of animals examined is too small and the variability too high to establish any exact correlation, but it is significant that in 70 per cent of the animals examined the gonads were 39 per cent underweight. A few of the animals whose growth record was described in the previous paper (1) but whose gonad data were not included in the table there given furnished figures similar to those given above and need not be added here.

In view of the contrast in reproductive behavior between the animals without and with the 5 per cent yeast addition it was surprising to find that the gonad weights of the latter differed so little from those just given. Of the nine females four showed an average underweight of 46 per cent and four an average overweight of 18 per cent. Thus 50 per cent of these animals had ovaries below normal weight as against 70 per cent in the animals without yeast addition. The figures on the four males with yeast addition are indifferent +12, +5, −4, and −15 per cent. They averaged 158 days of age when killed.

In this connection the gonad weights of the single surviving litter of six on milk-yeast ration[4] are of interest. The father of these animals was a stock male but they grew up on their mother's ration. At the age of 118 days when they were chloroformed the three males were 6 to 16 gm. underweight, the females were 18 to 24 gm. underweight. The ovary weights deviated −6, −7, and 0 per cent from the normal for that age. The testes weights deviated −47, −56, and −58 per cent. The males suffered less in body growth but much more in gonad development than the females.

Histological Material.

Sections of the gonads of ten males and six females were prepared and smears were made of testes contents, some of which were examined fresh, others were stained and preserved. Dr. Ezra Allen of Ursinus College kindly examined the testes sections and found them apparently normal with the exception of one in which the tubules were mostly in the state of degeneration described in his paper (9) as due to a lack of water-soluble vitamine and shown in his Fig. 8. The testis of this animal showed immature spermatozoa. "The heads are fairly well formed but the tubule has no lumen, is very narrow and shows some degeneration." This particular animal was 240 days old and had been on a 50 per cent milk ration until 181 days of age. Thereafter it had been fed the stock rat food. It was older than the others whose tissue Dr. Allen examined. The presence or absence of sperm in the smears of testis contents supports the idea that the obvious degenerative changes did not take place until sometime after maturity. The data on testes weights given above and the reproductive performance with stock female animals also bear this out. On combining the morphological and physiological data it appears that there was a partial fertility (partial because the offspring with normal females did not live) in males only to about 150 to 175 days of age. Such incipient infertility, if it might be so called, became manifest under the microscope as the animals grew older. Possibly the preparation of tissues so as to preserve cytological details might reveal degenerative changes preceding the more patent signs and the absence of sperm.

[4] Page 447.

The ovary slides were kindly examined by Dr. J. A. Long to whom we are also greatly indebted. Surprisingly, all of the ovaries appeared to be normal and indicated repeated ovulations. "In a few cases it is possible to state that ovulation has just taken place or is about to happen." At least two or three sets of corpora lutea were evident in all the ovaries, and no difference was apparent between animals on a milk ration and those on milk rations with added yeast, despite the fact that the latter cast litters and the former did not.

DISCUSSION.

The apparently normal ovaries in animals whose sole source of protein and vitamines was milk is not in agreement with the data of Evans and Bishop (5). Their dried milk was the same brand as ours (Merrell-Soule), but their milk rations contained milk only, without lard or starch additions, which is a possible explanation. As indicated above, the growth of animals on 100 per cent milk rations is inferior to that on lower percentages. It is entirely probable from several points of view, that the reproductive failure in our milk-fed female animals does not reside in the ovary. Quoting from the first paper of Evans and Bishop (10): "On the whole, good general growth or weight maintenance signifies a satisfactory ovulatory mechanism but not necessarily the ability to bear young,—that ovulation in fact, when compared with implantation and placental function is a relatively hardy mechanism and that much if not most sterility must be traced to uterine failure." The weight increments due to pregnancy in many of our experimental animals have often been smaller than the normal. Occasionally the curve ascended sharply as if gestation were beginning and in a few days descended again in a way not characteristic of normal fluctuations. If implantation is indicated by the initial increase in weight, the subsequent decline would indicate either abortion of which there has been no evidence, or the reabsorption of the embryo. We hope to obtain further evidence on this matter.

Beyond uterine failure there may also be a failure of lactation which was obvious in almost all of the animals that bore young and failed to raise them, and is not in harmony with the conclusions of Sugiura and Benedict (11). They found that milk

contains a substance (vitamine?) without which milk production did not occur, even though an adequate amount of vitamine B from yeast was present. This adequate amount was supplied by 0.5 per cent of dry yeast, a brewer's yeast which usually grew some mold during the process of drying in the laboratory; our rations contained 5 per cent of dry yeast (Fleischmann's without starch) and the drying was accomplished at low temperature (25–30°C.) without mold production. Yeast and milk evidently do not supply all the unknown requisites in adequate amounts. It may be assumed provisionally that a milk ration is inadequate for adult growth of the female rat, and especially for ovarian, uterine, and mammary functions; ovarian functions are the least disturbed of the three factors in reproduction. The addition of yeast tends to improve the uterine disability, but without making lactation successful. What the nature of this improvement is and what the other shortcomings of milk may be remain to be determined. According to Reynolds and Macomber (12) a moderate decrease in vitamine A, protein, or calcium materially decreased fertility in rats, and in discussing human infertility (13) they consider constipation as one of the factors. The last named is apparently the only one which might apply in this connection. In view of the very mild laxative action of yeast (14) this does not seem a likely explanation, but the alimentary tract of a grown rat may require bulk just as that of the adult human organism does. The addition of vitamine B to milk in order to secure successful lactation in rats has been tried with positive results by French (15) and Japanese (16) investigators, the latter using oryzanin. Excess protein (17) is probably not accountable for reproductive failure in our experiments.

With reference to the male animals it is apparent that on milk rations reproductive function is never entirely normal and that it suffers a progressive impairment which becomes complete toward 200 days of age. It is not clear from our experiments that the addition of yeast has any influence on the function except that in the second generation (the offspring of a stock male and an experimental female on yeast ration) the testes either entirely failed to develop or atrophied very early. Whether this is merely the result of milk alone or of added yeast remains to be determined. As evidence for the latter is the infertility

of animals whose sole source of protein and vitamine B was yeast (18); possibly yeast contains in addition to vitamine B some damaging substance whose presence is revealed when the amount used is large. The failure of animals to reproduce on synthetic rations in which all the vitamine B is supplied by yeast has recently been emphasized by Kennedy and Palmer (8) and experience with such rations in this laboratory is in accord with this statement.

The question is probably not one of vitamine B alone, but is one of supplementary relationships. The limiting dietary factors for successful reproduction in the rat appear not to be alike for both sexes and while these factors are still unknown a standardized synthetic ration for nutrition studies cannot be formulated.

Postmortem routine usually included examination of lungs, ears, and abdominal organs. The presence of pus in one or both middle ears and the extent of involvement of the lungs did not run parallel nor was there any relation between this and gonad weight. Thus of thirty-two animals whose ears were clean eleven showed an average figure of +19 per cent for the gonads, twenty-one an average of −37 per cent; of these twenty-one, eight also had absolutely normal lungs as far as could be determined and their average gonad weight was −35 per cent. The absence of any direct relation between the presence of infection and fertility has also been observed in stock animals provided there is no marked loss in weight.

SUMMARY.

On rations in which all the protein and vitamines were supplied by varying proportions of dry milk, rats (thirty-two males and twenty-two females) grew at a rate above the normal up to about 75 days of age. Thereafter the rate fell below normal, the decline in females being more rapid than in males. At 175 days females were 14 per cent underweight (average of thirty) and males 5 per cent underweight (average of sixteen). Rations containing 50, 60, and 70 per cent of dry milk were more favorable than 100 per cent. Reproduction was not successful on any of the rations and a change to rations containing added protein-free milk, cod liver oil, traces of KI, or even to stock rat food did not restore reproductive function. Mating tests with fertile stock animals

showed that milk-fed females were sterile, the males not always so although the offspring did not live. On milk rations (60 per cent) containing 5 per cent dry yeast, eight of nine females produced fourteen litters sired by males on the same ration; none of the young lived. One litter sired by a male on stock rat food did live. The success which other investigators have had in rearing two and more generations on all milk rations[1] cannot now be explained except by assuming that our colony is lacking in vigor and therefore responds more promptly to unfavorable circumstances.

From the functional tests, from the weight of the testes, and from histological examination of sections it is concluded that male rats on milk rations suffer a gradual decline in reproductive function which becomes complete toward 200 days of age. The testis shrinks to about half its normal size and, as examined by Dr. Ezra Allen, presents the appearance of degeneration described by him as resulting from an absence of water-soluble B. That the addition of yeast modifies this degeneration cannot as yet be stated definitely but it appears not to do so. The infertility of our female animals on milk rations cannot be explained by a decline in ovarian function since the ovaries, though only little more than half normal in weight, nevertheless, appeared normal in sections examined by Dr. J. A. Long. Ovulation seemed to be taking place frequently. The addition of yeast had no influence on the size of the ovaries and its influence, if any on uterine function, remains to be determined. The added vitamine B thus supplied failed to achieve normal lactation. There seems to be no relation between fertility and infection of the lungs and ears unless this is considerable.

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Addendum.—As this paper was going to press Evans and Bishop[5] announced the existence of a substance, X, found especially in green lettuce leaves, whose presence in the diet is necessary to secure normal reproduction. Placental rather than ovarian function was improved by its addition. Several of our observations are confirmatory of theirs and the assumption of still another unknown dietary requisite would perhaps explain the reproductive failure we have thus far not been able to correct on milk rations.

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THE RELATION OF LIPOIDS TO SUPRARENAL PHYSIOLOGY.

I. THE CHOLESTEROL AND LIPOID PHOSPHORUS CONTENTS OF THE BLOOD OF RABBITS BEFORE AND AFTER SUPRARENALECTOMY.

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As a result of a very large number of investigations published during the last two decades it has been held that the suprarenal gland, particularly the cortex, plays a prominent part in lipoid metabolism. Most of these investigations have been morphological in character and special reference is made to the doubly refractile bodies believed to be cholesterol esters. A few of the more recent papers have dealt with chemical aspects also, and an attempt has been made to correlate morphological with chemical results. The cholesterol and occasionally the total lipoid content of the suprarenal glands were considered, the material being mainly from human autopsies. Most investigators believe it is possible to obtain a rough idea of the total lipoid content of a gland from histological preparations stained with the usual fat stains, although Fex (1) in a very careful study concludes that the morphological and chemical data do not always correspond. Several authors have in addition maintained that the cholesterol ester content of the glands might be gauged from a study of frozen sections by means of the polarizing microscope.

Several investigators have noted that when cholesterol was fed to rabbits a decided increase in the total cholesterol content of the glands and of the blood occurs. These findings have been corroborated in this laboratory.

Grigaut (2), working with dogs, and Rothschild (3) with rabbits have followed the cholesterol content of the blood before and after unilateral, and the latter, also after bilateral, suprarenalectomy. Grigaut found that unilateral suprarenalectomy in dogs caused a gradual increase in the cholesterol content of the blood, followed by a fall to normal within 3 weeks after the operation. Rothschild obtained similar results with rabbits. He reports an even more marked and progressive hypercholesterolemia following double suprarenalectomy, continuing until the animals succumbed which, in his experiments, occurred usually within 24 hours.

From these and similar data Chauffard, Laroche, and Grigaut (4 to 8) and Chauffard, Richet, and Grigaut (9, 10) have postulated that cholesterol

is probably a substance of the hormone group and that it is manufactured by the suprarenal cortex. They interpret the hypercholesterolemia following unilateral suprarenalectomy in dogs as the result of a hypersecretion of the remaining gland.

Aschoff and his pupils have come to quite a different theory of the rôle of cholesterol in suprarenal physiology. They base their speculations largely on the experimental work of Rothschild (3) with suprarenalectomized rabbits, and on morphological examinations and chemical estimations of cholesterol content of suprarenal glands in various pathological states, by Landau and McNee (11)—data which are in many ways confirmations and extensions of those of Chauffard, Laroche, and Grigaut. As has already been stated, Rothschild in most, though not all, of his unilateral suprarenalectomized rabbits noted hypercholesterolemias quite similar to those reported by Grigaut working with dogs. However, when both glands were removed he found more marked hypercholesterolemias. On account of this observation he opposes the theory of Grigaut that the hypercholesterolemias following unilateral suprarenalectomy are due to an overactivity of the remaining gland, since it would be unlikely that the removal of an organ supposed to secrete cholesterol would cause an increase of cholesterol in the blood. Aschoff's followers have advanced the theory that the suprarenal gland is a storehouse for cholesterol acting in some intermediary capacity.

From this brief survey of the work on the rôle of the lipoids in suprarenal physiology it will be gathered that little is known except that the suprarenal gland, especially the cortical portion, is an organ that may contain a very high percentage of lipoids—as much as 20 per cent of the fresh weight. The fact that this organ normally has an unusually high cholesterol content, is not a sufficient basis for a theory that the suprarenal gland is the seat of production of cholesterol as the French investigators and others have maintained.

Likewise, the normally high cholesterol content of the suprarenals, the fact that feeding cholesterol further increases this store and that extirpation of these glands causes a hypercholesterolemia seems to us insufficient evidence on which to base a theory that the glands act as a storehouse for cholesterol. As will be shown later, lecithin, as determined by the lipoid phosphorus, undergoes changes strictly parallel to those found for cholesterol under the experimental conditions cited above. Lastly, the cholesterol content of the suprarenals is such a small fraction of the total present in the body that it could hardly be considered as a storehouse. The combined weight of both suprarenals of a rabbit varies from

about 0.3 to 0.7 gm. Even if they were to contain 10 per cent of cholesterol, an amount rarely present, they would not contain as much as is present either in the blood, brain, kidneys, liver, or most other organs.

The physiological significance of the hypercholesterolemia following suprarenalectomy in rabbits noted by Rothschild, might perhaps be questioned because all of his doubly suprarenalectomized animals died within 4 days or less. It is known that rabbits may survive bilateral suprarenalectomy for months and often indefinitely if proper surgical procedures and careful after treatment are used—facts amply confirmed upon large series of rabbits in this laboratory and elsewhere.

Bloor (12) and Terroine (13) have shown that during fat absorption and under other conditions where fat mobilization, infiltration, or depletion occurs, the cholesterol and lecithin content of blood undergo changes parallel in a general way with those of neutral fat. It would seem more reasonable in view of the foregoing presentation to assume that the rôle of cholesterol (and of the other lipoids) of the suprarenal glands might be a passive or an incidental one, yet possibly extremely important in suprarenal physiology. The present views are inadequate to explain the rôle of cholesterol in suprarenal physiology.

Marine and Baumann (14, 15) in studies on suprarenal insufficiency in rabbits, and Scott (16), working with cats, have shown that if a sufficient but sublethal deficiency is created such animals have an increased metabolic rate and that this depends upon the presence of iodine-containing thyroid glands (17, 18). As a tentative working hypothesis to explain the mechanism of this reaction they have attributed to the suprarenal cortex an inhibitory or regulatory influence over the thyroid and probably over other body activities also. From the work of Grigaut and Rothschild it seemed possible that cholesterol might play an important rôle in this inhibition. With this in mind it was decided to investigate this subject further, to repeat the work of Rothschild, avoiding criticisms already made, and to extend his work by including the estimation of lipoid phosphorus of the blood. At the same time the respiratory metabolism of the animals was determined to see if any relation existed between it and the blood lipoids; also to determine whether any suprarenal insufficiency had been created, and if so, to what extent.

Methods.

The animals used in this series of experiments were rabbits. Cholesterol and lipoid phosphorus determinations were made four or more times over a period of 2 weeks. The basal metabolism of the rabbits was determined during the period, though in a few cases the metabolic observations were not made until after the first suprarenal was removed. Marine and Baumann (14) have shown that removal of one gland does not as a rule, notably influence the metabolism of the rabbit. The animals were suprarenalectomized usually in two stages, but in one animal both glands were removed at one operation. In general cholesterol and lipoid phosphorus estimations as well as basal metabolism determinations were made three times a week after suprarenalectomy for a period of 3 or more weeks, and then twice a week until the animals died. In a few cases blood lipoid estimations were made daily for a short time after suprarenalectomy and in some animals that were apparently surviving double ablation indefinitely these determinations were made only once a week after a period of survival of 4 months. The rabbits were kept on a uniform diet of alfalfa, oats, and carrots. They were fed at 3 p.m. so that the respiratory exchange was determined 18 hours or more after food was given. The blood for lipoid determinations was obtained from the marginal ear vein about 18 hours after feeding. Adult rabbits withstand the loss of 4 cc. of blood thrice weekly for many months without any apparent ill effects. The lipoid estimations and respiratory exchange measurements were made on alternate days.

It is recognized that the animals were not in a postabsorptive state. It requires more than 2 days to get a rabbit into such a condition and such treatment is too severe to be used on a suprarenalectomized rabbit. It was hoped that a certain degree of uniformity could be obtained by using a regular diet and regular feeding time.

The respiratory exchange measurements were made with the Haldane open circuit apparatus as modified by Marine and Lenhart (19). Details of these measurements have been reported previously (15).[1]

[1] The respiratory exchange measurements were made by Miss Anna Cipra and Miss B. H. Lowe, to whom we wish to express our thanks.

The cholesterol estimations were made by the method of Myers and Wardell (20) which was found very satisfactory with a few minor modifications in the technical procedure. It is necessary to heat the blood after thoroughly mixing with plaster of Paris at 100–105°C. for about an hour. If lower temperatures were used the colors obtained on adding acetic anhydride and sulfuric acid did not compare well with the standards. The colors are best developed by placing in a bath at 22°C. in the dark. The standard used was made of pure cholesterol dissolved in chloroform. These solutions—stock and diluted ones—did not alter in composition in 6 months. They were kept in a refrigerator in glass stoppered bottles which were paraffined.

The lipoid phosphorus was estimated by a method similar to that recently published by Randles and Knudson (21); that is, a combination of the Bloor (22) extraction, and the oxidation and colorimetric procedures of Bell and Doisy (23). After using this procedure for some time, it was found that low results might be obtained due to volatilization and conversion of phosphoric acid in the oxidation process. A procedure to overcome these defects was developed (24). In view of the fact that we had begun to use the Bloor-Bell and Doisy adaptation and that the error was usually within 5 to 10 per cent it was decided to continue with it for the sake of uniformity. Briefly, the method was as follows:

1 cc. of blood was slowly added to about 20 or 40 cc. of alcohol-ether mixture in a 25 or 50 cc. graduate flask, the mixture brought to a boil in a water bath and immediately cooled, following exactly Bloor's (22) detailed directions. The mixture was diluted to the mark with alcohol-ether and filtered. An amount of filtrate equivalent to 0.4 or preferably 0.8 cc. of blood was evaporated to dryness in a large test-tube in a water bath after which 8 drops of concentrated sulfuric acid and 1 cc. of nitric acid were added. The nitric acid was slowly boiled off until white sulfur trioxide fumes were evolved and the solution was colorless, special care being observed to avoid heating the dry part of the tube in the last stages of oxidation. The contents of the tube were washed into a 25 cc. graduate with about 10 to 12 cc. of distilled water, and the color was developed by the method of Bell and Doisy except that a 10 per cent molybdate solution in 2 N sulfuric acid was used in place of the one they suggest. All apparatus must

TABLE I *

	Cholesterol	Lipoid phosphorus	Cholesterol	Lipoid phosphorus	Cholesterol	Lipoid phosphorus	Cholesterol	Lipoid phosphorus	Cholesterol	Lipoid phosphorus	Cholesterol.	Lipoid phosphorus
Class A.												
Rabbit No	262		274		276		304†		307		309	
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Average before right suprarenalectomy	61	5 7	65	7 1	68	6 8	64	7 4	69	6 6	64	7 0
Average 1st week after right suprarenalectomy	56	5 8			68	7 8			57	6 6	67	7 0
" 2nd " " " "	59	5 0	74	8 6	69	8 6			61	5 8	73	7 7
" 3rd " " " "			69	9 3	70	8 0			71	5 3	74	6 3
" 5th " " " "			68	7 8	70	8 3			67	9 0	66	5 8
" 7th " " " "			61	6 1								
" 1st " " left "	59	9 6	70	7 4	80	9 2	93	9 3	70	8 6	68	7 8
" 2nd " " " "	92	12 0	66	5 8	109	10 0	105	9 7	75	7 4	70	8 0
" 3rd " " " "			70	11 0			113	11 0	97	11 0		
" 4th " " " "			79	11 0			159		185	11 0		
" 5th " " " "			82				165	8 6				
Class B.												
Rabbit No	263		264		275		289		293		294	
Average before right suprarenalectomy	69	6 5	68	5 5	61	7 2	71	6 8	64	6 9	63	6 1
Average 1st week after right suprarenalectomy	58	6 1	54	5 0	64	8 0	74	5 6	62	6 5	58	6 9
" 2nd " " " "	60	6 4	54	6 4	70	9 1	69	7 9	61	8 0	61	9 3
" 3rd " " " "					72	8 3			63	8 1	65	10 0
" 5th " " " "									65	8 1	68	9.0

Average 1st week after left suprarenalectomy...	75	8.6	55	7.3	75	7.0	59	7.6	71	8.9	65	8.5
" 2nd " " " " ...	64	8.4	58	6.7	71	5.3	68	8.1	63	7.9	65	7.8
" 3rd " " " " ...	70	7.5	67	7.8	62	7.0	61	9.2	62	6.9	58	8.7
" 4th " " " " ...	69	9.0	66	7.7	66	6.7	65	8.2	54	6.6	57	6.7
" 5th " " " " ...	75	8.7	66	8.3	70	5.4	73	9.2	59	6.0	61	7.0
" 6th " " " " ...	78	7.9	78	8.9	65	5.6	75	9.7	61	5.4	67	6.0
" 7th " " " " ...	74	7.3	71	8.5	66	7.0	69	9.0		5.8	60	8.6
" 8th " " " " ...	79		68		65	7.3	69		59	7.4	59	8.3
" 9th " " " " ...	61	7.6	64	7.2	72	7.3	66	7.4	68	7.4	64	7.6
" 10th " " " " ...	63	8.7	65	7.1			73	7.4	61	8.8	59	8.1
" 11th " " " " ...	68	8.6	62	7.5			67	6.5	55	7.3	59	7.1
" 12th " " " " ...	57	6.0	60	5.2			70	6.7‡	69	8.9	79	11.0
" 13th " " " " ...			73	6.3			57	8.4	69	8.7	77	9.8
" 14th " " " " ...				7.1			79	10.0	68	8.9	76	10.0
" 15th " " " " ...			66	7.0			66	9.5	68	8.8	79	9.0
" 16th " " " " ...			60	7.4			64	8.2	71	9.9	88	12.0
" 17th " " " " ...			56				72	8.9	76	10.0	98	11.0
" 18th " " " " ...							68	8.1	78	10.0		
" 20th " " " " ...							80	9.4	74	9.6		
" 22nd " " " " ...							75	8.7				
" 24th " " " " ...							79	9.4				
" 26th " " " " ...							95	9.8				
" 28th " " " " ...							85	11.0				

* All data are given in mg. per 100 cc. of blood.

† Right and left suprarenals removed Dec. 1, 1921.

‡ Two accessory suprarenals removed with testes.

be thoroughly washed with distilled water before being used and all reagents except the carbonate-sulfite solution must be tested for phosphates.

Presentation of Data.—Seventeen rabbits were used in this series of experiments, of which twelve were suprarenalectomized. Of the twelve, one (No. 276) died of pneumonia 12 days following the removal of the second suprarenal. The others may be grouped into classes: Class A, those dying within 4 or 5 weeks; and Class B, those living for 4 or 5 months, or indefinitely. The condensed data of these experiments are presented in Table I. In the first class there were five rabbits. In these animals little or no accessory suprarenal tissue was found. It has been shown by many observers that the duration of life of suprarenalectomized rabbits is roughly proportional to the amount of accessory tissue present. In some the metabolism increased; in others it fell somewhat, especially in the last week of life. That is, they belonged to Groups II and III, respectively, in the classification of Marine and Baumann. The protocol of Rabbit 307 is given in Table II as typical of this class.

In the second class there were six rabbits. Rabbit 263 died 12 hours after an uncomplicated ovariectomy and 81 days after its suprarenals had been removed. Rabbit 294 was sacrificed 17 weeks after suprarenalectomy because of a clinical diagnosis of a chest infection which proved to be empyema. Rabbits 293 and 275 were sacrificed 29 weeks after suprarenalectomy to terminate the experiments and were apparently in excellent condition. Rabbits 264 and 289 died of chronic suprarenal insufficiency 17 and 28 weeks, respectively, after the second suprarenal had been removed. The protocols of Rabbits 264 and 293 are presented in Tables III and IV as typical of this class. The metabolism of all these animals rose after suprarenalectomy from 15 to 80 per cent above their average normal values; they belong therefore in Group II in the classification of Marine and Baumann (14).

The normal range of cholesterol and lipoid phosphorus content of the blood for these seventeen rabbits on a standard diet is summarized in Table V. All these cholesterol and most of the lipoid phosphorus estimations were made in duplicate, separate determinations on each animal were made on different days and where four or more estimations were made on the same animal

TABLE II.

Rabbit 307.

Date.	Weight of rabbit.	Calories per kilo per hour.	Cholesterol per 100 cc.	Lipoid P per 100 cc.
1921	*gm*		*mg.*	*mg.*
Dec. 15	2,289	2.61*	74	8.5
" 20	2,371	2.58*	75	7.4
1922				
Jan. 27	2,333	2.53	60	
Feb. 3	2,368	2.63	60	7.8
" 7	2,367	2.61	53	5.2
" 10	2,347	2.53	77	7.5
" 14	2,409	2.42		
" 17	2,392	2.50	70	5.3
" 20	2,382	2.32		
" 24	2,402	2.64	65	8.8
" 27	2,372	2.31		
Mar. 6	2,399	2.52	66	9.0
" 10	2,340	2 33	68	
" 12	2,371	2 42		
" 14	2,296	2.46	71	8.6
" 17	2,242	2.52	73	
" 21	2,185	2.34	76	7.3
" 24				8.8
" 28	2,119	2.46	96	13.1
" 31	2,022	2.27	140	11.1
Apr. 3	1,835	2.07*	229	8.9

* The dates given are those on which the blood lipoid estimations were made. The metabolism observations were made on the day previous except those marked with an asterisk where observations were all made on the same day.

Protocol 1.—Rabbit 307. Female, white adult. Jan. 13, 1922. Under ether removed right suprarenal completely. Weight 0.17 gm. Abundant abdominal fat. Mar. 7. Under ether removed left suprarenal completely. Weight 0.25 gm. Kidney apparently normal. Mar. 17. Gave 25 mg. of KI by mouth. Mar. 28. Eating only a small amount of carrot. Apr. 1. Continues to eat very little; losing weight. Apr. 3. Dead 1 hour after being run for metabolism. Very weak when removed from chamber. Necropsy at once. Thyroids pale and translucent—iodized. Thymus atrophic. Heart muscle flaccid. Lungs edematous. Abdominal organs all markedly reduced in size except ovaries which were perhaps slightly enlarged. Stomach contained a large ulcer nearly healed and many pin-point ulcers. Moderate amount of abdominal fat. No accessory suprarenals found.

TABLE III.

Rabbit 264.

Date.	Weight of rabbit.	Calories per kilo per hour.	Cholesterol per 100 cc.	Lipoid P per 100 cc.
1921	*gm.*		*mg.*	*mg.*
Aug. 10	2,590	2.03*		
Sept. 20	2,580	1.89*		
" 23	2,622	2.12*		
" 26	2,602	2.06*		
Oct. 12			62	4.7
" 14			71	6.6
" 18			64	5.9
" 31			60	5.3
Nov. 2			79	4.7
" 4				5.4
" 7			54	4.5
" 10			54	6.1
" 14			54	6.6
" 16			58	7.4
" 18			61	6.9
" 20			52	7.5
" 23			58	6.7
" 26				6.6
" 29			67	7.5
Dec. 1	2,748	3.03*	65	8.0
" 6			52	6.1
" 9	2,820	2.85*		
" 10		*	78	9.2
" 13	2,821	3.48*	56	6.9
" 15	2,864	2.96*	74	9.7
" 19	2,833	3.10*	65	8.4
" 23	2,869	3.27*	77	8.9
" 30	2,842	3.27*	70	8.5
1922				
Jan. 5	2,858	2.96*	67	
" 11	2,923	2.54*	63	7.7
" 14	2,832	2.96*	63	6.6
" 17	2,736	2.86*	69	6.8
" 20	2,805	2.96*	61	7.4

* The dates given are those on which the blood lipoid estimations were made. The metabolism observations were made on the day previous except those marked with an asterisk where observations were all made on the same day.

TABLE III—*Concluded.*

Date.	Weight of rabbit.	Calories per kilo per hour.	Cholesterol per 100 cc.	Lipoid P per 100 cc.
1922	*gm.*		*mg.*	*mg.*
Jan. 25	2,880	2.95	57	7.0
" 28	2,906	2.88	67	8.0
Feb. 1	2,973	2.67	58	6.4
" 4	2,881	2.90	60	
" 7	2,842	3.25	71	6.1
" 10	2,842	2.74	74	6.4
" 14	2,859	2.50		6.9
" 17	2,858	2.51		7.2
" 21	2,810	2.38	64	7.5
" 24	2,861	2.39	67	6.4
" 28	2,879	2.27	59	7.7
Mar. 3	2,962	2.34	57	8.3
" 6	2,895	2.82	62	6.0
" 10	3,024	1.92	56	
" 13	2,918	2.93		

Protocol 2.—Rabbit 264. Male, gray adult. Nov. 2, 1921. Under ether removed right suprarenal completely. Weight 0.18 gm. Nov. 14. Under ether removed left suprarenal completely. Weight 0.24 gm. Very little abdominal or perirenal fat. Kidney appears normal. Nov. 16. Feels warm. Stools soft. Eating large ration. Nov. 19. Eating about 1.5 times the usual ration. Nov. 29. Unusually sexually active. Has become vicious. Dec. 8. Gave 25 mg. of KI by mouth. Dec. 20. Definite sweating noted on shaved areas. Continues to eat large extra ration. Increased sexual activity continues. Jan. 18, 1922. Losing weight; added corn to ration. Feb. 20. Not so active. Eats full ration; possible atrophy of testis. Mar. 5. More active again. Mar. 11. Eating extra ration completely; active. Mar. 14. Died last night. Was run for metabolism the afternoon of Mar. 13th and did not eat anything when removed. Evidently effect of stay in metabolism chamber on heart led to death. *Necropsy.*—Thyroids possibly slightly enlarged, vascular, no visible colloid; contained less than 0.01 mg. of iodine per gm. of dry tissue. Parathyroids hyperemic. Thymus present; fair size. Lungs congested and edematous, no consolidation. *Heart.*—Right side dilated; left contracted. Right and left suprarenals absent. A 2 mm. accessory in right epididymis and possibly one in left epididymis. Spleen enlarged. Pancreas hyperemic. Stomach autodigested. Kidneys large and had few pittings on surface. Testes soft. All cutaneous vessels markedly dilated and distended with blood. Moderate amount of abdominal and subcutaneous fat; very hyperemic. Death clearly connected with previous metabolism run; cardiac weakness and exhaustion secondary.

TABLE IV.

Rabbit 293.

Date.	Weight of rabbit.	Calories per kilo per hour.	Cholesterol per 100 cc.	Lipoid P per 100 cc.
1921	*gm.*		*mg.*	*mg.*
Oct. 10			72	5.6
" 14			59	8.5
Nov. 28			60	7.6
" 30			63	5.9
Dec. 1			68	6.0
" 2	2,486	3.02*	51	6.2
" 3			63	6.3
" 5	2,486	2.93*		
" 7	2,472	3.05*	54	7.2
" 9	2,428	2.72	60	7.9
" 10	2,468	2.66*		
" 13	2,473	2.64*		
" 16	2,445	2.80*	59	6.8
" 21	2,484	2.68*	66	9.4
" 30	2,472	2.64*	66	8.5
1922				
Jan. 3	2,466	2.60*	64	7.7
" 6	2,413	2 51*	65	
" 9	2,389	2.92*	76	8.9
" 12	2,300	2.56*	66	7.6
" 16	2,295	2.96*	59	8.1
" 19	2,312	2.86*	65	6.8
" 25	2,363	2.78	57	
" 28	2,416	2.63	54	6.6
Feb. 1	2,463	3.02		6.6
" 4	2,488	3.00	57	7.4
" 7	2,484	3.14	60	4.4
" 10	2,495	3.62	60	5.2
" 14	2,481	3.00		5.4
" 17	2,466	2.79		7.3
" 21	2,472	2.96		4.2
" 24	2,497	3.11	57	8.2
" 28	2,486	2.77	59	6.5
Mar. 3	2,488	3.42	61	

* The dates given are those on which the blood lipoid estimations were made. The metabolism observations were made on the day previous except those marked with an asterisk where observations were all made on the same day.

TABLE IV—*Concluded.*

Date.	Weight of rabbit.	Calories per kilo per hour.	Cholesterol per 100 cc.	Lipoid P per 100 cc.
1922	*gm.*		*mg.*	*mg.*
Mar. 6	2,478	3.43	74	7.3
" 10	2,528	3.40	59	
" 14	2,528	3.08	63	8.8
" 17	2,510	3.02	56	
" 21	2,503	2.82	53	7.3
" 24	2,528	3.36	66	8.6
" 28	2,458	3.19	71	9.1
" 31	2,482	3.55		8.7
Apr. 4	2,433	3.24	69	
" 7	2,503	2.86	67	8.9
" 11	2,514	2.83		8.8
" 14	2,525	3.08	71	10.0
" 18	2,508	2.65	64	7.6
" 21	2,503	2.64	72	9.9
" 25			69	9.9
May 2			76	10.1
" 9	2,505	2.72	77	10.0
" 16	2,508	2.81	72	7.8
" 24			73	9.6

Protocol 3.—Rabbit 293. Male, young gray adult. Nov. 29, 1921. Gave 25 mg. of KI by mouth. Repeated dose Nov. 30 and Dec. 8. Nov. 30. Under ether removed right suprarenal completely. Weight 0.13 gm. Jan. 4, 1922. Under ether removed left suprarenal completely. Weight 0.21 gm. Kidney normal. Abdominal fat abundant. Jan. 9. Stitches torn out probably in handling. Resutured. Jan. 21. Soft stools. Eating full ration. Feb. 14. Coat seems especially clean and bright. July 8. Under ether removed right and left testes. Large accessory suprarenal in left testis. On section found a 2 mm. accessory in each spermatic cord near testis. July 13. No ill effects from gonadectomy. Sacrificed at 2 p.m. Weight 2,341 gm. *Necropsy.*—At once. Gonadectomy wound rapidly healing. Other wounds healed in January. Thyroids very hyperemic. Parathyroids pale and small. Thymus present; large and cellular. Heart in systole; appears normal. *Lungs.*—Small patches of consolidation in upper lobes. In addition to the two accessory suprarenals removed with testes, a 2 mm. accessory found on left side above site of left suprarenal. Pancreas hyperemic. Slight pittings on surface of kidneys. Abdominal fat scant.

they were done over a period of 2 or 3 weeks. The 58 normal cholesterol estimations range from 54 to 86 mg. per 100 cc. of blood with an average of 67 mg. per 100 cc. In one rabbit a value

of 45 mg. per 100 cc. was found. The figure is unusual and was not included in the averages. For a given animal the variation on different days was rarely more than 20 mg. per 100 cc. and usually less. The lipoid phosphorus values showed greater fluctuations, from 4.8 to 8.7 mg. per 100 cc., the average being 6.5 mg. per 100 cc. Here the range for a given animal was rarely more than 2.0 mg. per 100 cc. These figures are probably low for reasons already set forth. However, they are believed to be of value in indicating any significant changes due to experimental procedures.

TABLE V.

Normal Variations of Blood Lipoids.

Sex.	Rabbit No.	Cholesterol per 100 cc.			Lipoid P per 100 cc.		
		Maximum.	Minimum.	Average.	Maximum.	Minimum.	Average.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
M.	262	74	56	62 (5)	6.1	5.1	5.7 (5)
F.	263	81	67	70 (5)	6.9	5.9	6.5 (5)
M.	264	80	60	68 (5)	6.6	4.8	5.5 (5)
"	274	62	62	62 (1)			
"	275	46	45	46 (2)			
F.	283	83	63	73 (2)	5.8	5.1	5.4 (3)
M.	289	75	65	71 (4)	7.8	5.4	6.8 (4)
"	293	72	59	64 (4)	8.5	5.7	6.9 (4)
"	294	77	55	63 (4)	6.5	5.8	6.1 (4)
F.	304	67	62	64 (3)	7.9	6.6	7.4 (4)
"	307	76	61	71 (4)	8.5	5.7	7.2 (4)
"	309	86	70	79 (2)	7.5	6.5	6.9 (3)
M.	288	74	57	66 (3)	7.8	5.0	6.3 (3)
"	291	81	60	72 (3)	6.4	5.6	6.0 (3)
?	292	79	60	72 (4)	6.5	5.6	6.1 (4)
F.	303	76	54	69 (4)	8.0	7.0	7.3 (3)
"	305	77	70	73 (3)	8.7	6.1	7.7 (3)
Average.	17	74	60	67	7.3	5.7	6.5

Figures in parenthesis indicate number of estimations.

DISCUSSION.

Unlike Rothschild we observed no increases in the cholesterol content of the blood following removal of one gland. On the contrary, in the ten uncomplicated experiments six animals actually showed a temporary lower blood cholesterol value, the decrease amounting to about 15 per cent of their preoperative averages,

while four showed no unusual variations. The blood cholesterol returned to normal within a week.

The lipoid phosphorus remained unchanged following the removal of one suprarenal in six of the ten experiments, but in four, increases varying from 15 to 50 per cent occurred. These variations were not outside the "normal" range and could not be correlated with the cholesterol results just described. With the facts at present available these changes are not looked upon as being especially significant.

In animals that survived double suprarenalectomy for 5 or more weeks there were no unusual changes in the blood cholesterol during the first 4 weeks. The lipoid phosphorus values, however, increased definitely in all cases during the first fortnight and perhaps even more markedly during the next fortnight. That these changes are not due to the incidental operative procedure or to the anesthesia is shown by two types of control experiments: (1) simple anesthesia; and (2) manipulating the intestines. The latter procedure is more shocking than the operation of suprarenalectomy. The fact that no such changes occurred after the removal of one gland in six of ten cases would also indicate that the changes in lipoid phosphorus of the blood are not associated with the operative method used.

The terminal effects of double suprarenalectomy were, among other things, a general rise in both the cholesterol and lipoid phosphorus content of the blood. This usually occurred during the last 2 to 6 days of life, although in Rabbit 304 this rise lasted for 30 days. It is to be noted that this is the only instance in which such a prolonged rise was observed. It happens to be the animal in which both the glands were removed at one operation.

These terminal rises are similar to those noted by Rothschild except that in his experiments all animals except one lived less than a day after removal of the second suprarenal, and the one that lived 4 days was found to have a large accessory. In the experiments reported here, the period of survival was never less than 17 days. In part, at least, this rise is believed to be due to blood concentration. Unfortunately no direct measurements of blood concentration were made. The following data, however, show that other constituents of the blood increased during the last week in rabbits dying of suprarenal insufficiency.

Rabbit No.	Date.	Cholesterol per 100 cc.	Lipoid P per 100 cc.	Urea N per 100 cc.	Creatinine per 100 cc.
	1922	*mg.*	*mg.*	*mg.*	*mg.*
262	Oct. 18	59.4	5.9	12.3	1.2
262	" 23	103	11.8	96.6	9.0
275	Jan. 6	134	11.9	86.1	4.5

It is not believed that the terminal lipoidemias following suprarenalectomy have any physiological significance which is necessarily connected with suprarenal function. By the time the hypercholesterolemias appear, the animals are suffering from far more generalized effects than suprarenal insufficiency. Many, perhaps most, body activities are profoundly depressed by the time the blood lipoids increase, and this lowering of vitality is sufficient to account for the accumulation of various metabolites in the blood. It is not improbable that such animals are moribund.

As a rule, though not invariably, the fluctuations in the lipoid phosphorus and cholesterol of the blood were roughly parallel. The lipoid phosphorus changes were greater in extent and often reached beyond the normal range, while the changes in the cholesterol values were within normal limits except during the terminal rise.

No constant relation of blood lipoids to respiratory metabolism was observed. However, there is considerable literature suggesting that such a relation does occur. Vernon (25) and others have suggested that tissue oxidation is connected in some way with its lipoid content. More recently, Epstein and Lande (26) have expressed their belief in an inverse relation of blood cholesterol values to basal metabolism, especially in patients that have Graves' disease; that is, when the metabolism is high, cholesterol values will be low, and conversely.

In our experiments no evidence tending to verify any of these relationships was obtained. Occasionally during the period of increased metabolism the blood lipoids were high. More often they were high during periods of normal or subnormal metabolism. Sometimes in the same animal during periods of increased, normal, or decreased metabolism the blood lipoids were high (Rabbits 289, 293, and 294), while in one animal especially, Rabbit 264, the curves of basal metabolism and blood lipoids were strikingly parallel.

These data in so far as they go lend no support to Vernon's hypothesis, although they furnish no argument against it, since little oxidation probably occurs in the circulating tissue. However, if Epstein's hypothesis is correct, our data should offer evidence in support of it. The animals were kept under conditions much more readily controlled than is possible with hospital patients and wide variations in their rate of heat production were observed. As pointed out by Marine and Baumann suprarenal insufficiency in rabbits is in many ways similar to the symptom complex of Graves' disease; yet in this very extensive series of observations no relationship between metabolism and cholesterol or lipoid phosphorus could be established. It is possible that the low cholesterol content of blood often present in Graves' disease is dependent upon the total lipoid content of the blood. As Bloor and Terroine have shown, changes in the total fatty acids of the blood are roughly paralleled by changes in the lipoid phosphorus, and in a lesser degree, by the cholesterol contents of the blood. In cases of Graves' disease with high basal metabolism there is an increased demand for fuel and little tendency to adiposity; therefore, the blood of such patients would be apt to be low in lipoids. Luden and others have published observations showing increased blood cholesterol in myxedema. It is probable because of the increased total fatty acids and lipoid phosphorus that the cholesterol values are also increased. There is no necessary relationship between blood cholesterol and the plane of metabolism.

We wish to express our thanks to Dr. David Marine for the assistance and helpful criticism that he has given throughout this work.

CONCLUSIONS.

1. There is no evidence for the hypothesis of Grigaut that the suprarenals are the seat of cholesterol formation. Nor is there reason at present to believe that these glands act as a storehouse for cholesterol.

2. The cholesterol and lipoid phosphorus content of blood were studied before and after unilateral and bilateral suprarenalectomy in twelve rabbits. There was no significant change in the blood

cholesterol until the last week of life, when it rose. The lipoid phosphorus increased occasionally after unilateral and invariably after bilateral suprarenalectomy. A terminal rise was also noted as in the case of cholesterol. This terminal lipoidemia is best explained as a premortal phenomenon.

3. No direct relation between the plane of metabolism and the blood lipoids could be established.

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INVESTIGATIONS ON THE IMMEDIATE EFFECT OF HEAVY EXERCISE (STAIR-RUNNING) ON SOME PHASES OF CIRCULATION AND RESPIRATION IN NORMAL INDIVIDUALS.

II. OXYGEN AND CARBON DIOXIDE CONTENT OF BLOOD DRAWN FROM A CUBITAL VEIN AT DIFFERENT INTERVALS AFTER EXERCISE.

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INTRODUCTION.

In a previous investigation[1] it was shown that, in sixteen out of seventeen normal individuals, the oxygen content of blood drawn from a cubital vein decreased markedly immediately after leg exercise (fast stair-running five times). The samples of blood analyzed were drawn within 1 minute after the exercise was finished. The experiments presented in this paper were undertaken in order to ascertain how soon the values for the oxygen saturation of the venous blood come back to normal. From one to three samples of blood were drawn from a cubital vein at different intervals after cessation of exercise. During that period, the subject remained quiet on a bed. The technique and experimental conditions are fully described in Paper I.[1]

EXPERIMENTAL RESULTS.

Oxygen.—In Table I six experiments are reported. The time is given only approximately; it corresponds to the middle of the period during which the blood sample was drawn. The drawing of the first sample after exercise was always finished within

[1] Lundsgaard, C., and Möller, E., *J. Biol. Chem.*, 1923, lv, 315.

1 minute after the exercise (stair-running) was finished. In five out of six experiments the oxygen content of the blood from a cubital vein immediately after exercise was very low, ranging

TABLE I.

Oxygen and Carbon Dioxide Content of Venous Blood Drawn from a Cubital Vein before and at Different Intervals after Leg Exercise (Stair-Running Five Times).

Experiment No.	No. in protocol.	Name.	Conditions under which blood sample was drawn.	Total oxygen-combining power of blood.	Oxygen content of venous blood drawn from arm.	Oxygen unsaturation of venous blood drawn from arm.	Carbon dioxide content of venous blood drawn from arm.	Pulse rate.	Respiration rate.
				vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*		
1	A 11	E. M.	Rest.		16.73	3.82	48.56	80	14
			½ min. after exercise.	20.55	9.45	11.10	51.78	120	24
			4 " " "		19.75	0.80	46.90	96	18
			8 " " "	20.55	18.03	2.52	45.57	84	15
2	A 19	S.	Rest.	20.95	14.03	6.92	44.10		
			½ min. after exercise.	21.20	7.43	13.77	47.63	132	36
			7 " " "		10.04	11.16	45.91	72	16
3	A 23	E. M.	Rest.	19.82	16.75	3.07	50.68	76	18
			½ min. after exercise.		5.98	13.84	57.14	120	32
			6 " " "		17.02	2.80	50.54	84	20
4	A 25	H. S.	Rest.		15.16	4.86	57.61	60	14
			½ min. after exercise.	20.02	5.37	14.65	48.20	148	28
			6 " " "				37.92	84	20
5	A 26	H. S.	Rest.	19.51				70	14
			½ min. after exercise.		4.01	15.50	47.71	140	32
			6 " " "	19.51	13.83	5.68	41.27	72	26
6	A 20	C. L.	Rest.		16.42	3.20	51.39	74	16
			½ min. after exercise.	19.62	16.87	2.75	43.30	120	40
			8 " " "	19.80	18.76	1.04	38.16	72	15

from one-fifth to one-half of the total oxygen-combining power of the blood. Experiment A 20 (Table I) differed from the first five experiments, showing a normal value half a minute after exercise. The subject on whom this experiment was done is the same one (C. L.) on whom we performed a number of experiments reported in Paper I of this series (No. 17), all of which showed

TABLE II.

Oxygen Content of Venous Blood Drawn from the Arm before and at Different Intervals after Leg Exercise (Stair-Running Five Times).

Subject E. M. Experiments B 32 and B 33.

Experiment No.	No. in protocol.	Name.	Conditions under which blood sample was drawn.	Total oxygen-combining power of blood.	Oxygen content of venous blood drawn from arm.	Oxygen unsaturation of venous blood drawn from arm.	Pulse rate.	Respiration rate.
				vol. per cent	*vol. per cent*	*vol. per cent*		
1	B 32	E. M.	Rest.	19.54	14.89	4.65		
			70 to 120 sec. after exercise.	19.72	12.23	7.49	152	36*
2	B 33	E. M.	Rest.	19.01	12.66	6.35		
			42 to 82 sec. after exercise.	18.65	8.48	10.17	160	30*

* Pulse and respiration rates immediately after exercise.

TABLE III.

Oxygen Content of Venous Blood Drawn from the Arm during Rest and at Different Intervals after Leg Exercise (Stair-Running Five Times).

Subject E. P. Experiment B 30.

Time.			Condition.	Pulse.	Respiration.	Sample of venous blood drawn from arm.			
Hour.	Minute.	Second.				No.	Total oxygen-combining power.	Oxygen content.	Oxygen unsaturation.
							vol. per cent	*vol. per cent*	*vol. per cent*
2	8	00	Rest.						
2	21	00		76	20				
2	21	40	Bleeding.			1	19.28	16.80	2.83
2	22	38							
2	26	40	Exercise.						
2	28	08							
2	28	35	Bleeding.	150	44	2	(19.28)	9.77	9.86
2	28	52							
2	30	40		116					
2	31	00			24				
2	33	10		112					
2	33	40	Rest.		24				
2	36	38	Bleeding.	100		3	19.98	17.05	2.58
2	37	20							
2	37	40			16				
2	39	40		80					
2	40	43	Bleeding.			4	(19.98)	17.18	2.45
2	41	38							

high values for oxygen immediately after exercise. In samples of blood drawn from 4 to 8 minutes after exercise, we found in all six experiments either normal values or values higher than those normally found. In Experiment A 11 (Table I), a higher value was found 4 minutes after exercise than was found 8 minutes

TABLE IV.

Oxygen Content of Venous Blood Drawn from the Arm during Rest and at Different Intervals after Leg Exercise (Stair-Running Five Times).

Subject E. P. Experiment B 31.

Time.			Condition.	Pulse.	Respiration.	Sample of venous blood drawn from arm.			
Hour.	Minute.	Second.				No.	Total oxygen-combining power.	Oxygen content.	Oxygen unsaturation.
							vol. per cent	*vol. per cent*	*vol. per cent*
2	7	00							
2	22	00	Rest.	80	20				
2	22	37							
2	23	02	Bleeding.			1	20.24	18.76	1.34
2	23	38							
2	25	08	Exercise.						
2	25	24							
2	25	48	Bleeding.	141	25	2	(20.24)	4.35	15.75
2	26	58							
2	27	54	Bleeding.	116	16	3	19.97	16.84	3.26
2	28	40							
2	29	32	Bleeding.			4	(19.97)	18.31	1.79
2	29	55	Rest.	96					
2	30	35			20				
2	31	08		100					
2	34	45		88					
2	40	52		91					

after. Corresponding to this, we observed that the blood drawn after 4 minutes was almost as red as fully oxygenated blood.

In later experiments given in Tables II to IV, the exact time of the drawing of the blood was noted. In the experiments reported in Table II, a lower oxygen content was found in a sample drawn from 42 to 82 seconds after exercise (Experiment B 33) than in another sample drawn from 70 to 120 seconds after

exercise (Experiment B 32). Both these experiments were done on a subject on whom several experiments (No. 2, Table I, Paper I) had been previously performed. In Experiments B 30 and B 31 (Tables III and IV), both done on the same subject (E.P.), results were obtained similar to those given in Table II. Samples drawn later than 1 or 1½ minutes after the exercise gave values for the oxygen content of venous blood higher than, or equal to, those usually found at rest. As in Experiment A 11 (Table I), the

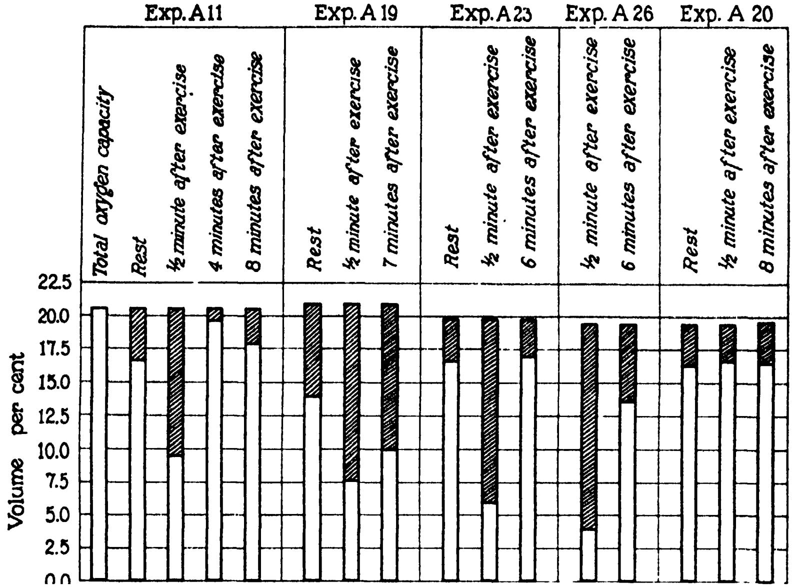

FIG. 1. Diagram representing the results of the experiments on oxygen given in Table I. Experiment 4 (A 25) is not included in the diagram.

The height of the columns indicates the total oxygen-combining power of the hemoglobin. The shaded area represents the deoxidized fraction of the hemoglobin and the plain area the oxidized fraction.

oxygen content seemed to be at its maximum about 2 to 3 minutes after exercise, containing almost as much oxygen as is found in normally oxygenated arterial blood. In interpreting the results of these experiments, it must be borne in mind that the oxygen content of peripheral blood drawn during rest from the same vein may normally vary over a wide range.

All the results are given diagrammatically in Figs. 1 and 2, which need no further explanation. In Fig. 3, we have constructed a curve on the basis of the two experiments (Nos. B 30 and B 31, Tables III and IV) performed on the same subject. We have, furthermore, plotted on the diagram the values for the oxygen content immediately after exercise in Experiments B 32 and B 33 (Table II). The curve shows clearly that the period following exercise (stair-running) during which one may obtain

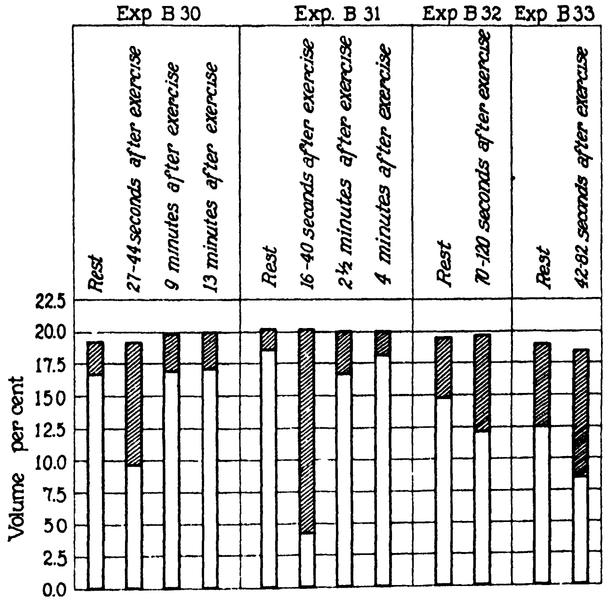

Fig. 2. Diagram representing the results of the experiments on oxygen in Tables II, III, and IV.

The height of the columns indicates the total oxygen-combining power of the hemoglobin. The shaded area represents the deoxidized fraction of the hemoglobin and the plain area the oxidized fraction.

unusually low values for oxygen in the venous blood, is of very short duration after exercise, about 1 to 1½ minutes. After this comes a period when the blood oxygen may be very high, almost as high as that found in arterial blood, and later on "normal" values are obtained. The two figures from Table II, plotted on the diagram in Fig. 3, correspond very closely to the values from Experiments B 30 and B 31 (Tables III and IV).

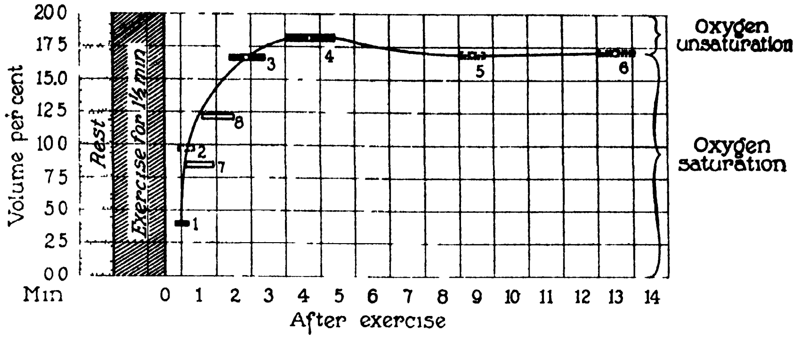

FIG. 3. Areas 1 to 8 indicate the determinations of the saturation of the hemoglobin in the experiments in Tables II, III, and IV. Their distance from the abscissæ represents the degree of oxygen saturation. The length of these areas indicates the exact time and duration for the drawing of the blood sample. The time after the cessation of the exercise is given on the abscissæ. A curve is drawn through the six points (1 to 6) representing determinations on the same individual (E. P.).

Areas 1, 3, and 4 are from Experiment B 31. Areas 2, 5, and 6 are from Experiment B 30. Areas 7 and 8 represent determinations on another individual (E. M., Experiments B 32 and B 33). The values for the first series of experiments given in Table I are not plotted as the exact time (in seconds) for the beginning of the drawing of the blood was not noted.

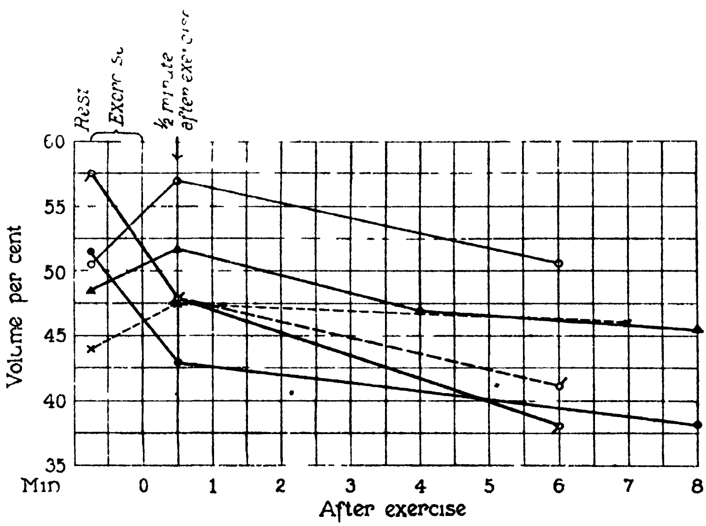

FIG. 4. Carbon dioxide content of venous blood at rest, ½ minute after exercise, and 4 to 8 minutes after stair-running. (Compare Table I.)

Carbon Dioxide.—Determination of the carbon dioxide content of the venous blood was done only in the six experiments reported in Table I. Similar to our findings in the earlier experiments (Paper I), the values for carbon dioxide, in some instances (two experiments) decreased immediately after exercise, in other instances (three experiments) an increase was found. If, however, the values for the carbon dioxide content of the blood, drawn ½ minute after exercise, are compared with the values in samples drawn later, a marked decrease is seen to take place during the first minutes after exercise (Fig. 4).

DISCUSSION.

We shall not enter into a discussion of the underlying causes of the changes in the oxygen content of the blood from a cubital vein after stair-running. A preliminary discussion was given in Paper I, and before further conclusions can be drawn, we want to present additional experiments. The decrease in carbon dioxide content is probably due to accumulation in the blood of fixed acids (lactic acid) produced in the muscles of the lower extremities during exercise. The fact that the carbon dioxide content is lower 4 to 6 minutes after exercise than immediately after, is probably caused by a late appearance of acid products in the general circulation, corresponding to what has been found by Lindhard[2] after static exercise. Lindhard found that the carbon dioxide output and the oxygen intake increased markedly after cessation of static exercise. During the same period, a decrease in the (reduced) pH took place. From the results of his experiments, Lindhard draws the conclusion that the statically contracted muscles offer a mechanical hindrance to the (local) circulation. The metabolism, therefore, becomes to a great extent anaerobic, on account of an insufficient supply of oxygen during exercise. He furthermore concludes that an accumulation of lactic acid takes place in the active muscles during static exercise, and he considers this responsible for the early and pronounced fatigue in such cases. It seems possible that during heavy leg exercise (fast stair-running) the oxygen supply of the active muscles is insufficient, so that conditions may prevail similar to those which,

[2] Lindhard, J., *Skand. Arch. Physiol.*, 1920, xl, 145.

according to Lindhard, are present during and shortly after static exercise. We noticed that the fast stair-running used in our experiments made the subjects feel very tired for some time (usually 5 to 15 minutes) after the exercise was over.

SUMMARY.

1. The period during which the oxygen content of blood drawn from a cubital vein is found to be low after fast stair-running five times, is of very short duration (about 1 minute after the exercise is finished). 2 to 4 minutes after exercise, venous blood drawn from the arm may contain almost as much oxygen as arterial blood. From 5 to 8 minutes after exercise the value again decreases somewhat and becomes more like the "normal average."

2. The carbon dioxide content of the blood decreases markedly (5 to 10 volumes per cent) during the first few minutes after the exercise is finished.

THE ACTION OF AMMONIUM CYANIDE ON DIKETONES.

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Strecker's (1) synthesis of amino-acids based upon the addition of hydrocyanic acid to aldehyde ammonia compounds and hydrolysis of the resulting amino nitrile has proved one of the most useful general methods of amino-acid synthesis. Tiemann's (2) modification of the reaction involving the formation of amino nitriles by the addition of the ammonia to the aldehyde or ketone cyanhydrin extended the utility of the reaction considerably. The direct addition of ammonium cyanide to aldehydes and ketones as first used by Ljubavin (3) and later used by many others particularly Zelinsky and Stadnikoff (4), who used mixtures of potassium cyanide and ammonium chloride in aqueous solution, furnished a variant of the method which offers a particularly easy method of obtaining amino nitriles and hence of amino-acids of widely varying types.

In connection with the synthesis of certain homologues of diaminosuccinic acid the action of ammonium cyanide upon benzil, the simplest aromatic α-diketone, was studied. It was anticipated that diphenyldiaminosuccinic acid would result from the following series of reactions:

$$C_6H_5 \cdot CO \cdot CO \cdot C_6H_5 \rightarrow C_6H_5 \cdot C(NH_2)\,CN \cdot C(NH_2)\,CN \cdot C_6H_5$$
$$\rightarrow HOOC \cdot \underset{\substack{| \\ C_6H_5}}{C}\,NH_2 \cdot \underset{\substack{| \\ C_6H_5}}{C}\,NH_2 \cdot COOH$$

It was found, however, that the reaction took an entirely different course and that not more than a trace of amino-acid was formed, and even this trace did not have the anticipated structure. On investigation it was found that benzil on treatment with ammonium cyanide was almost quantitatively resolved into benzamide and benzaldehyde cyanhydrin:

$$C_6H_5 \cdot CO \cdot CO \cdot C_6H_5 + NH_4\,CN \rightarrow C_6H_5 \cdot CONH_2 + C_6H_5 \cdot CHOH \cdot CN$$

On hydrolysis, the products gave benzoic and mandelic acids together with vanishingly small amounts of phenylaminoacetic acid.

The reaction seemed sufficiently peculiar to warrant a little further investigation. On starting with benzil dicyanhydrin and acting upon this with alcoholic ammonia precisely the same reaction took place with formation of benzamide and benzaldehyde cyanhydrin, hence it is inferred that the failure of benzil to undergo what may be regarded as the normal amino nitrile synthesis is not likely to be due to inability on the part of ammonium cyanide to effect the introduction of two (CN) groups.

Derivatives of benzil were found to react with ammonium cyanide in an analogous fashion. For example, anisil gave anisamide (*p*-methoxy-benzamide) and some anisaldehyde cyanhydrin. Piperil gave a good yield of piperonylamide and piperonal cyanhydrin, while *m*-dinitrobenzil gave *m*-nitrobenzamide together with an oil which, however, contained none of the expected nitrobenzaldehyde cyanhydrin, but was a condensation product resulting from the interaction of *m*-nitrobenzaldehyde cyanhydrin with unchanged dinitrobenzil in the presence of ammonia. The action of ammonium cyanide on furil, the α-diketone derived from furfural, was also examined. Pyromucamide together with an oil yielding furfural on distillation from alkaline solution were obtained in good yield, showing that the same type of reaction had occurred as had been observed in the case of benzil.

An examination of the literature showed that with one very significant reaction described by Ljubavin (5) the action of ammonium cyanide on α-diketones and related substances had been scarcely investigated. Ljubavin found that glyoxal on treatment with ammonium cyanide gave among other products an amino nitrile which was converted into an amino-acid on hydrolysis. At first Ljubavin described the amino-acid as the then unknown diaminosuccinic acid, assuming that the normal reaction had taken place, but later he found that the substance was simply glycine. There can hardly be any doubt but that the glyoxal underwent a similar reaction to that now observed with benzil, giving primarily formamide and formaldehyde cyanhydrin, and that the latter by the further action of ammonium cyanide gave

aminoacetonitrite which, as is well known, yields glycine on hydrolysis:

$$CHO \cdot CHO \rightarrow H \cdot CO \cdot NH_2 + H_2COH \cdot CN$$

$$CH_2OH \cdot CN \rightarrow CH_2 \cdot NH_2 \cdot CN \rightarrow CH_2 \cdot NH_2 \cdot COOH$$

A few other substances structurally related to benzil and glyoxal were examined as regards their reaction with ammonium cyanide without disclosing further examples of the new type of reaction, but on the other hand, no α-diketone was found to undergo the normal amino-acid synthesis of Strecker and Tiemann. Diacetyl, $CH_3 \cdot CO \cdot CO \cdot CH_3$, reacted vigorously with ammonium cyanide but on hydrolysis the products contained neither lactic acid, alanine, nor dimethyldiaminosuccinic acid. The bulk of the product appeared to be glyoxaline derivatives. Phenylglyoxal, $C_6H_5 \cdot CO \cdot CHO$, was completely converted into glyoxaline derivatives without any trace of amino-acid formation. Benzoin, $C_6H_5 \cdot CHOH \cdot CO \cdot C_6H_5$, and dibenzalacetone, $C_6H_5 \cdot CH{:}CH \cdot CO \cdot CH{:}CH \cdot C_6H_5$, were practically unchanged by ammonium cyanide in aqueous alcohol, even on warming. Benzoylacetone, $C_6H_5 \cdot CO \cdot CH_2 \cdot CO \cdot CH_3$, was mostly unchanged but on hydrolysis gave a small amount of some tarry unidentified substance. There was no indication either of amino-acid synthesis or of a fission of the chain.

The disruption of the carbon chain in glyoxal and benzil under the regulated action of aqueous ammonium cyanide is sufficiently remarkable to make it desirable to correlate it with other known reactions. It may be recalled that Debus' (6) original synthesis of glyoxaline from glyoxal and ammonia depends on the resolution of a molecule of glyoxal into formaldehyde and formic acid, of which only the former product participates in completing the ring synthesis. Jourdan (7) showed that benzil gave benzaldehyde and benzoic acid when warmed with sodium carbonate and a little potassium cyanide while Michael and Palmer (8) found that benzil was stable when heated with alcohol at 200°, but that if a little hydrocyanic acid was added benzaldehyde and ethyl benzoate were formed. The formation of ethyl benzoate in the preparation of benzil dicyanhydrin by the action of hydrocyanic acid on alcoholic solutions of benzil has also been referred to by Japp and

Miller (9). The significance of these reactions to the one under discussion requires no further comment.

From the foregoing results it is concluded that the action of ammonium cyanide upon α-diketones does not lead to amino nitriles giving amino-acids on hydrolysis. In some cases complex reactions with formation of glyoxaline derivatives take place while in others fission of the carbon chain occurs between the two carbonyl groups.

EXPERIMENTAL.

Action of Ammonium Cyanide on Benzil.—The following conditions were found suitable for carrying out the reaction. Benzil (2 gm.) suspended in 2 cc. of ether is placed in a corked cylinder. Ammonium chloride (1.5 gm.) together with 1 cc. of water is then added. To the mixture, a solution of sodium cyanide (1.5 gm. in 3 cc. of water) is added by degrees with shaking and cooling under running water. After half an hour the mixture is shaken out with ether and the ether extract allowed to evaporate. The residue comprises a mass of benzamide crystals together with oily benzaldehyde cyanhydrin. They are conveniently separated by washing with a little petroleum in which the benzamide is almost insoluble. The benzamide weighed 1.0 gm., and on crystallization from dilute alcohol it melted sharply at 128°. It contained 11.4 per cent nitrogen compared with a calculated value of 11.5. The oily cyanhydrin (1.1 gm.) contained 10.2 per cent of nitrogen (calculated 10.5 per cent), and on hydrolysis with hydrochloric acid gave mandelic acid which was recovered by extraction with ether. On recrystallizing the mandelic acid from benzene it was readily purified and melted sharply at 116–117°.

Preparation of Benzildicyanhydrin.—The following method was found preferable to that of Japp and Miller (9), since it avoids the use of alcohol, which has a deleterious action on the product. Benzil is dissolved in three times its weight of benzene and a large excess of anhydrous hydrocyanic acid added. The homogeneous mixture is allowed to stand. If crystallization does not occur after a couple of days the addition of an equal volume of benzene will affect it. The dicyanhydrin is almost insoluble in benzene and separates out in colorless plates in practically quantitative yield. It is a curious fact that the product which first separates

has a very much higher melting point than that hitherto ascribed to the compound. In one case the melting point was over 180°. On standing, the melting point is slowly depressed and eventually approximates that usually given for the substance; namely, 132°. The high melting product on analysis gave 10.24 per cent nitrogen (calculated 10.6 per cent). It seems reasonable to assume that two isomeric cyanhydrins are produced of which the low melting variety rarely is the more stable. No evidence was obtained indicating any chemical difference between the two varieties.

Action of Ammonia on Benzildicyanhydrin.—The powdered cyanhydrin (2 gm.) was mixed with methyl alcoholic ammonia equivalent to 2½ molecular proportions. It dissolved readily and darkened in color. After standing over night the alcohol was allowed to evaporate, leaving a crystalline substance mixed with an oil. These proved to be benzamide and benzaldehyde cyanhydrin which were separated and identified in the same fashion as that adopted in the case of the products from benzil. The benzamide melted at 128° and the mandelic acid from the benzaldehyde cyanhydrin melted at 116–117°.

Action of Ammonium Cyanide on m-Dinitrobenzil.—The dinitrobenzil for these experiments was prepared as follows: Benzil (3.12 gm.), finely powdered, was added gradually to a mixture cooled to $-5°$ of sulfuric acid (25 cc.) and finely powdered ammonium nitrate (10 gm.). The whole was allowed to stand in an ice bath for 3 hours with occasional stirring. On pouring the mixture onto ice, a yellow flocculent precipitate of the nitro compound separated which was filtered off and on recrystallization from alcohol gave 2 gm. of the pure *m*-dinitrobenzil melting at 108°. This method has certain advantages over that described by Klinger and Martinoff (10), which requires the use of anhydrous nitric acid. The reaction with ammonium cyanide was carried out as follows: The powdered dinitrobenzil (1.5 gm.) was added to a mixture of alcohol (2 cc.), water (3 cc.), and ammonium chloride (0.8 gm.). A solution of sodium cyanide (0.7 gm.) in water (2 cc.) was then added with cooling and shaking. The mixture darkened rapidly and was allowed to stand for 15 minutes. It was then extracted with ether. On evaporation of the ether a large separation of nitrobenzamide occurred which, after washing with benzene, was recrystallized from boiling water. It weighed 0.6 gm. and

melted at 140–142°. On hydrolysis it gave *m*-nitrobenzoic acid. The oil recovered from the benzene mother liquor gave no nitromandelic acid on heating with hydrochloric acid and was practically insoluble in aqueous acid. On heating with sodium hydroxide it was readily decomposed and on acidifying, hydrocyanic acid and a high melting unidentified glyoxaline derivative were obtained in large amount. It is probable that the oil resulted from the condensation by ammonium cyanide of *m*-nitrobenzaldehyde or its cyanhydrin with unchanged dinitrobenzil. Analogous condensations to give lophine derivatives are well known. Special experiments with pure *m*-nitrobenzaldehyde showed that it reacted normally with ammonium cyanide to give cyanhydrins which on hydrolysis gave nitromandelic acid in excellent yield together with small amounts of nitrophenylaminoacetic acid.

Action of Ammonium Cyanide on Anisil.—Anisil was obtained by the action of Fehling's solution on anisoin according to the method described by Biltz and Wienands (11). The finely powdered substance (2 gm.) was mixed with ammonium chloride (1.5 gm.), water (3 cc.), and alcohol (2 cc.). An aqueous solution of sodium cyanide (1.5 gm.), dissolved in water (4 cc.), was added and the whole vigorously shaken with the addition of ether. The reaction takes place much more slowly than in the case of benzil or dinitrobenzil and requires several hours before the whole of the anisil is decomposed. The ether solution on evaporation gave a pasty mass of crystals which were washed with benzene. The crystals weighed 0.85 gm. and were pure anisamide. On recrystallization from water colorless needles melting at 164° were readily obtained (nitrogen, 9.33 per cent found, 9.27 per cent calculated). The benzene mother liquor was evaporated at a low temperature. The residue contained anisaldehyde and its cyanhydrin. The aldehyde was readily identified by its phenylhydrazone, melting at 120–121° after crystallization from benzene. Since the complete hydrolysis of anisaldehyde cyanhydrin is difficult to effect smoothly, the remaining oil was mixed with concentrated hydrochloric acid and allowed to stand in the cold as described by Tiemann and Köhler (12). The crystalline residue (0.27 gm.) was washed with ether and identified as *p*-methoxymandelamide, melting at 159° after crystallization from alcohol. The formation of this substance is conclusive evidence of the formation of ani-

saldehyde cyanhydrin in the original reaction with ammonium cyanide.

Action of Ammonium Cyanide on Piperil.—Piperil was obtained from piperoin by oxidation with Fehling's solution as described by Biltz and Wienands. The reaction with ammonium cyanide was rather slow, requiring about 3 hours. The conditions of the experiment were identical with those adopted in the case of anisil. The ether extract from 2 gm. of piperil on washing with benzene gave 0.9 gm. piperonylamide (nitrogen, 8.39 per cent found, 8.48 per cent calculated). On recrystallization from water it melted sharply at 169°. The benzene mother liquor on hydrolysis gave a small yield (0.15 gm.) of methylene-3-4-dihydroxymandelic acid melting at 154°, but as Lorenz (13) has observed, much decomposition takes place in the reaction. The properties of the acid were identical with those described by Lorenz. There is, therefore, no doubt of the formation of piperonal cyanhydrin together with piperonylamide by the action of ammonium cyanide on piperil and the reaction is entirely analogous to that observed in the case of benzil.

Action of Ammonium Cyanide on Furil.—The furil was prepared by Fischer's method (14) by oxidizing an alkaline solution of furoin with air. Furil (2 gm.) was mixed with ether (4 cc.), ammonium chloride (1 gm.), and sodium cyanide (1 gm.), dissolved in water (6 cc.). The reaction takes about 2 hours for completion. The ether layer was then separated and the aqueous layer extracted once with fresh ether. The pyromucamide was chiefly in the aqueous layer and was readily obtained pure by repeated extraction with butyl alcohol. On evaporation of the solvent at a low temperature, 0.8 gm. of pyromucamide melting at 142–143° was obtained. An additional 0.12 gm. was obtained from the ether layer so that the yield was very good. (Nitrogen, 12.2 per cent found, 12.4 per cent calculated.) On evaporation of the ether layer an oil was obtained together with a little crystalline pyromucamide which is sparingly soluble and readily separated. On attempting to hydrolyze the oil with acid it was converted into a black pitchy mass. It was therefore distilled with dilute aqueous sodium carbonate when more than half a gram of furfural was obtained. The aldehyde was identified by the usual reactions and by its phenylhydrazone, melting at 98°. It should be noted

that furfural cyanhydrin appears not to have been described, hence it appeared preferable to convert the oil into furfural which is so readily identified.

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STUDIES IN THE PHYSIOLOGY OF MUSCULAR EXERCISE.

I. CHANGES IN ACID-BASE EQUILIBRIUM FOLLOWING SHORT PERIODS OF VIGOROUS MUSCULAR EXERCISE.

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When one considers the great interest which has recently been evinced in the subject of acidosis, it is somewhat surprising to find that the most frequent cause of acidosis has received little attention. During and following vigorous muscular exercise, the acid-base equilibrium of the blood undergoes changes which are comparable in degree to those observed in severe diabetes or nephritis, and which may occur often in the daily life of any active, healthy individual. The investigation reported in this and the following papers is concerned with the direction, extent, and duration of these changes.

Comparatively few studies of the acid-base equilibrium of the blood have been made during or after exercise. The reasons are not hard to find. Venous blood from the arm or other available surface veins may give little reliable information concerning conditions of the mixed venous blood and still less concerning the arterial blood supplying the tissues. The unreliability of venous blood and the difficulty and supposed danger of obtaining arterial blood from man have led most observers in the past to focus their attention upon the air from the deep parts of the lungs with the assumption that this air is in gaseous equilibrium with the circulating blood in the pulmonary capillaries. Although this mode of approach is indirect, much valuable and probably quite accurate

information has been obtained concerning gas tension and reaction of blood under constant resting conditions. When these methods are applied to muscular exercise, however, the difficulties increase. Until such fundamental questions as the diffusion coefficient of gases through lung membranes and the secretion of gases by the lungs have been definitely settled, the relationship of the blood gases to the gases in the alveolar air must remain open to doubt. Furthermore, there is uncertainty concerning our ability to determine or to calculate correctly the composition of deep lung air during exercise. The method of Haldane and Priestley (1) gives percentages which are admittedly too high (2), the exact amount of the error being subject to dispute. The calculation employed by Krogh and Lindhard (3) to estimate the composition of alveolar air depends upon the correct determination of the dead space, the volume of which during exercise is a subject of vigorous controversy.

Although the chief interest in the subject has been centered upon the indirect respiratory methods, study of the blood itself has not been entirely neglected. In 1888, Geppert and Zuntz (4) published results of experiments on the effects of muscular work produced by tetanizing the hind limbs of an animal after section of the spinal cord. They found a marked decrease in the carbon dioxide content of arterial blood following the tetanus. Hastings (5) demonstrated a reduction in the carbon dioxide-combining capacity of venous plasma in dogs which had been doing heavy muscular work on a treadmill. Christiansen, Douglas, and Haldane (6), studying the carbon dioxide absorption curve of a man after muscular exercise, found a diminution of 40 per cent in the carbon dioxide-combining capacity of whole venous blood. In one experiment, Harrop (7) found a reduction in the carbon dioxide content of both arterial and venous blood in a man following fatiguing exercise. Lindhard (8) found a lowered combining capacity in two subjects after static work upon the horizontal bars.

Direct determinations of carbon dioxide tensions of arterial and venous blood during exercise have not been made. There are a few scattered observations upon the hydrogen ion concentration. In the above mentioned experiments of Lindhard, the pH was determined by the method of Krogh and Liljestrand (9). A

reduction of 0.05 to 0.06 in pH was demonstrated 1 to 2 minutes after the exercise had ceased. In a climb of 1,000 feet up Carlingford Mountain, Barcroft (10) found changes in the oxygen dissociation curve of his finger blood which indicated a fall from his normal pH 7.29 to 7.09 after the climb. Parsons, Parsons, and Barcroft (11), studying the finger blood of Barcroft, found by measurements with the hydrogen electrode a fall in pH of 0.08 during the last moments of moderate work on a bicycle ergometer.

One fact has been firmly established by these studies of blood. The carbon dioxide content and capacity are greatly reduced after work. It would appear also from the few observations which have been quoted, that the reaction of the blood becomes less alkaline. Haldane (12), however, believes, from the evidence of his experiments on alveolar air and pulmonary ventilation, that the change in reaction is much less than the above figures would indicate.

The uncertainty of respiratory methods and the many gaps in our knowledge concerning the composition and reaction of the blood itself, have suggested the present investigation. Stadie's (13) demonstration of the safety of arterial puncture and Haggard and Henderson's (14) method for the direct determination of carbon dioxide tension of arterial and venous blood have made comparatively simple a research which a few years ago would have been fraught with the greatest difficulties.

Procedure and Methods.

For the measurement of work, we used a Krogh bicycle ergometer which Dr. F. G. Benedict was kind enough to lend us. All experiments were done during the morning from 1 to 2 hours after an ordinary breakfast. No attempt was made to control the diet. The breakfast of the subject (D. P. B.) on whom the largest number of observations were made was always the same and consisted of fruit, four slices of toast, two eggs, and coffee. Before the first blood sample was taken the subject rested for 30 minutes in a steamer chair.

Collection of Blood.—In the first few experiments, arterial blood was obtained from the radial artery. In the later observations, it was found more convenient and less distressing to the patient to use the brachial. Venous blood was taken from an arm vein without pressure. The samples were drawn in 20 cc. syringes in which had been placed about 2 cc. of sterile white mineral oil to protect the blood from contact with air. As soon as drawn, the blood was transferred rapidly into mercury collecting tubes

which contained the anticoagulant. Preparation of the collectors consisted in introducing 0.25 cc. of a 20 per cent solution of neutral potassium oxalate which was dried on the walls of the tube without heat by means of a current of air. The amount of oxalate was calculated to furnish a concentration of 0.25 per cent in 20 cc. of blood.

Analyses.—Upon each sample of blood determinations were made of the carbon dioxide and oxygen content and of the carbon dioxide capacity or absorption curve. The latter was obtained by exposing samples of the blood in a water bath at 37.5°C. to carbon dioxide-air mixtures containing known amounts of carbon dioxide. In the early experiments, oxygen capacity was determined only once, the blood before and after exercise being used interchangeably for this purpose. Later, the value for oxygen capacity was obtained on each specimen. The analyses for carbon dioxide and oxygen were made in large Van Slyke pipettes by the methods described by Van Slyke and Stadie (15). All analyses were made in duplicate by two observers working simultaneously. Lactic acid content was determined by the method of Clausen (16).

Equilibration.—With certain modifications, the first saturation method of Van Slyke and his associates (17) was used for the equilibration of blood. The tonometers were of approximately 500 cc. capacity and were equipped at both ends with two-way stop-cocks. The small vessel (described in Van Slyke's method) for receiving blood after equilibration was not used. After 20 minutes of horizontal rotation in the water bath, the tonometer was held perpendicularly to allow the blood to collect at one end. It was then removed from the bath and connected with a mercury collecting tube to which the blood was rapidly transferred. The gas mixture which still remained in the tonometer was analyzed for its exact percentage of carbon dioxide. From this, the carbon dioxide tension was calculated since the gas mixture in the tonometer was equilibrated with atmospheric pressure at room temperature, a correction was made for the alteration in carbon dioxide tension which was caused by the higher temperature in the water bath. Further correction was made for vapor pressure.

This technique was not entirely satisfactory because the blood was exposed during the transfer for a brief interval to small differences in temperature and pressure. After several observations had been made, we performed the following experiment. Two samples of the same blood were exposed to equal carbon dioxide tension and equilibrated for an equal time in the water bath. One sample was then transferred to the mercury collector in the manner described above. Into the upper stop-cock of the other tonometer, about 2 cc. of mineral oil were injected and allowed to form a layer above the blood before removal of the tonometer from the bath. The blood was then drawn into the mercury collector in the usual way. The blood which was transferred without oil contained 50.7 volumes per cent of carbon dioxide, while the sample on which the oil had been layered contained 50.1 volumes per cent. Although no considerable error was demonstrated, it was believed that the use of oil eliminated the possibility of loss or gain of carbon dioxide after the removal of the blood from the

water bath. This precaution was observed in all experiments which were performed after Mar. 1, 1922

Method of Representation.—The CO_2 diagrams, introduced by Haggard and Henderson (14), were used for the graphic representation of the results. Corrections for oxygen unsaturation of arterial and venous blood were made by the formula[1] of Peters, Barr, and Rule (19). The hydrogen ion concentration, expressed as pH, was calculated from the carbonic acid-bicarbonate ratio, using a pK_1 of 6.10. The CO_2 tension of arterial and venous blood was read off directly on the diagrams after corrections for oxygen saturation had been made.

Sources of Error.

Analysis.—The errors inherent in the methods of analysis for CO_2 and oxygen have been fully discussed by Van Slyke and Stadie (15). In the succeeding paragraphs such errors as may have arisen from our procedure will be considered. Fortunately, these errors affect chiefly absolute values and have little or no influence upon the conclusions, drawn from a comparison of conditions before and after exercise.

Formation of Acid in Blood.—Christiansen, Douglas, and Haldane (6) showed that when blood was allowed to stand, its carbon dioxide capacity gradually diminished, indicating the formation of acid in blood. Peters, Barr, and Rule (19) observed a similar change, but found that no significant changes occurred during the 1st hour that blood was allowed to stand at room temperature. In the course of the present experiments it was shown repeatedly that, as long as the blood was kept in the mercury receiving tubes, successive samples removed for analysis contained the same amount of carbon dioxide. This does not preclude the possibility of acid changes but demonstrates that blood, which is kept in such a way that no carbon dioxide can escape, will reveal no change in carbon dioxide content by analysis. The values for carbon dioxide content of the blood as drawn were, therefore, not affected by acid formation. Errors were introduced, however, when the blood which had been standing in the collectors was removed for equilibration. Any acid formation which might have occurred within the tubes reflected itself in a reduction of the carbon dioxide-combining capacity. Furthermore, the exposure of the blood to a temperature of 37.5°C. increased and accelerated the change. The length of time in which the blood stood at room temperature varied in our experiments from 30 to 90 minutes. The time of exposure in the water bath was always 20 minutes.

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[1] Doisy, Briggs, Eaton, and Chambers (18) have recently published work in which they question the value of the empiric factor used by Peters, Barr, and Rule in correcting for oxygen unsaturation. Their experiments indicate that this should be 0.27 instead of 0.34 which was used in these experiments. If this should prove to be correct, it would change slightly our values for pH and CO_2 tension in the venous blood but would exert no demonstrable effect on the results in arterial blood.

Since the samples of blood taken before and after exercise were treated simultaneously and in exactly the same manner, it was believed that any error which might arise from acid formation would be approximately the same before and after exercise. Absolute values might not be correct, but the comparison of conditions should be valid. The timely appearance of Evans' (20) paper afforded us an opportunity of testing this assumption. Evans found that by the use of small amounts of sodium fluoride, the acid formation in blood *in vitro* could be checked. With this knowledge the following experiments were performed.

Blood was drawn from an arm vein before and after exercise. One-half of each sample was delivered into mercury receivers containing neutral potassium oxalate and was allowed to remain at room temperature. The other half was delivered into similar tubes which contained neutral 0.1 per cent sodium fluoride in addition to the oxalate. These tubes were immediately placed in a freezing mixture and after a few minutes were removed to an ice bath. All the samples were allowed to stand for 1½ to 2 hours and were then equilibrated with equal tensions of carbon dioxide in the water bath at 37.5°C. The experiments were performed in midsummer when the greatest acid production might have been expected. The results are given in Table I. The samples which were not protected with fluoride had a lower carbon dioxide capacity indicating acid formation during the time of standing and equilibration. The degree of change in each blood, however, was essentially the same before and after exercise.

The experiments demonstrate that acid formation introduces no considerable error in the interpretation of comparative results. In addition to this control, eighteen experiments were done after the appearance of Evans' work in which use was made of sodium fluoride to protect the blood (see protocols of this and the following papers of the series). The results were indistinguishable from those which had been done previously. None of the conclusions which had been drawn from the original experiments was altered.

Calculation of pH.—While the calculation of pH from the carbonic acid-bicarbonate ratio rests upon a sound theoretical basis, the absolute values obtained in this manner are subject to serious doubt. The calculation from Hasselbalch's (21) formula depends upon the value of a constant which was determined empirically upon plasma. Parsons (22) questioned the applicability of a constant which was obtained from a single phase system like plasma to whole blood in which there are two phases, cells and plasma. In a comparison of hydrogen ion concentration measured electrometrically with that calculated from the carbonic acid-bicarbonate ratio in whole blood, he found differences which indicated that the constant of Hasselbalch, 6.10, was too low. The difference averaged from 0.03 to 0.04 and in one instance was as great as 0.09. In more recent experiments Barcroft and his associates (23) obtained by similar comparisons results which indicate a constant of 6.17 to 6.19. Van Slyke, Austin, and Cullen (24) found in the blood of several dogs an average value of 6.20. The constants recently

obtained by Warburg (25) in a most comprehensive study correspond closely to those of Van Slyke and his associates. Although all the recent evidence seems to indicate that Hasselbalch's constant and consequently his figures for pH are 0.05 to 0.10 too low, we have adhered to the probably faulty value, 6.10, until more consistent and complete information is forthcoming.

Occasional Sources of Error.—In those individuals upon whom several experiments were performed the CO_2 absorption curve was not always determined. The CO_2 capacity was obtained at one tension only and curves were constructed from this single point, using the slope of the curve previously determined. This may have introduced small errors in the calculation of pH and CO_2 tension in a few of the experiments.

TABLE I.

Effect of Time on the CO_2-Combining Capacity of Blood with and without 0.1 Per Cent Sodium Fluoride.

Subject.	Date.	Time at room temperature	Room temperature.	Time in water bath at 37.5°C.	CO_2 capacity at 40 mm.					
					Before exercise.			After exercise.		
					With sodium fluoride.	Without sodium fluoride.	Difference.	With sodium fluoride.	Without sodium fluoride.	Difference.
	1922	*min.*	*°C.*	*min.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
D. P. B.	June 9	120	29	20	47.0	46.0	1.0	35.0	34.2	0.8
H. E. H.	" 13	90	21	20	50.8	50.0	0.8	40.2	39.3	0.9
M. F.	" 23	120	22	20	47.8	45.6	2.2	36.2	34.2	2.0

Analysis for oxygen capacity was made only once in many of the earlier experiments, the blood before and after exercise being used interchangeably for the purpose. Since the oxygen capacity is slightly increased during and after exercise, a small error was thus introduced in the estimation of pH and CO_2 tension through faulty correction for oxygen unsaturation. This did not amount to more than 0.01 in pH or 1 mm. in CO_2 tension. In the later experiments oxygen capacity was determined separately for each specimen of blood.

In the protocols at the end of this and the succeeding papers of the series, one may see the experiments in which these sources of possible error were present.

Direction and Extent of Error.—The absolute values for pH in all of our experiments may be 0.05 to 0.10 too low depending upon the exact value for the constant in Hasselbalch's equation. A further reduction in pH values may have occurred as a result of acid changes in those experiments

in which the blood was not protected by sodium fluoride The greatest observed change in CO_2-combining capacity from this cause was 2.2 volumes per cent. If this had occurred in all experiments it would have caused us to calculate our pH values about 0.05 too low and the CO_2 tension approximately 5 mm. too high. The actual error introduced from this cause was probably much less in most instances, since the results which were obtained in the same individual with and without fluoride were practically indistinguishable.

The errors which have been discussed affect only absolute values for CO_2 tension and pH and are of no importance when the figures are used for the comparison of conditions before and after exercise as they are in the succeeding discussion.

Complete protocols of the experimental data will be found at the end of Papers I, II, and III of this series. In the discussion, the experiments are referred to by initials and date.

Changes in Carbon Dioxide Absorption Curve, Carbon Dioxide Tension, and Reaction of Blood 3 Minutes after Exercise.

The first group of experiments was designed to demonstrate the changes occurring in the blood of several young adult men. Preceding the protocols are brief descriptions of the subjects. All were without organic defect. None of them was in training. N. P. L. had been an athlete and was in excellent physical condition at the time of the test. Three subjects were in poor condition. P. R. was 3 days convalescent from an attack of acute tonsillitis. J. E. was studied 10 days to 2 weeks after a prolonged alcoholic debauch. K. G. H. had never completely recovered his strength following an attack of encephalitis lethargica in 1919. The others were students, internes, and teachers leading sedentary lives without regular exercise. Each subject did approximately 3,500 kilogrammeters of work in $3\frac{1}{2}$ minutes. An approximate equivalent of this task would be accomplished if a 150 pound man were to climb stairs to the height of 150 feet at the rate of one step per second. The work was accomplished by lifting a weight of 2.5 kilos at the rate of about 200 revolutions per minute. The speed was not strictly controlled so the actual amount of work varied slightly. In those subjects, upon whom one set of observations was made, the variation was from 3,300 to 3,800 kilogrammeters. Included in the group, are three observations upon one subject, H. E. H., who on the different occasions did

3,055, 3,545, and 3,954 kilogrammeters. Blood specimens were taken immediately before exercise and about 3 minutes after the work had ceased. The second sample was started at 2½ minutes and was usually complete at the end of 3 to 3½ minutes. Ten experiments were done upon arterial blood, the results of which are tabulated in Table II. Fig. 1 is a graphic representation of a typical experiment.

TABLE II.

Changes in CO_2-Combining Capacity, CO_2 Tension, and Reaction of Arterial Blood after Exercise.

Subject.	Date.	Amount of work.	CO_2 capacity at 40 mm.			CO_2 tension of arterial blood.			pH		
			Before.	After.	Difference.	Before.	After.	Difference.	Before.	After.	Difference.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
	1922	*kgm.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*mm. Hg*	*mm. Hg*	*mm. Hg*			
M. L.	Apr. 7	3,285	46.2	31.9	14.3	47.5	44 5	3.0	7.28	7.11	0.17
N. P. L.	Nov. 15	3,595	46 3	34.7	11.6	43.2	40.2	3.0	7.30	7.17	0.13
J. McL.	" 30	3,605	48.4	32.6	15.8	45.2	39.5	5.7	7.30	7.15	0.15
P. R.	Dec. 10	3,695	45.0	26.2	18.8	41.3	40.0	1.3	7.29	7.04	0.25
J. E.	Nov. 2	3,700	42.7	27.9	14.8	42.0	30.5?	11.5?	7.28	7.12?	0.16?
H. B. R.	Apr. 12	3,500?	46.8	35.7	11.1	43.0	36.5	6.5	7.30	7.22	0.08
D. P. B.*	Aug. 8	3,770	49.2	32.9	16.3	36.5	23.0	13.5	7.36	7.27	0.09
H. E. H.	Mar. 29	3,055	48.4	40.9	7.5	46.0	40.7	5.3	7.29	7.25	0.04
H. E. H.*	Aug. 25	3,545	47.9	39.3	8.6	46.5	44.0	2.5	7.28	7.21	0.07
H. E. H.	Apr. 5	3,954	50.2	32.5	17.7	49.0	38.3	10.7	7.29	7.15	0.14

* Sodium fluoride, 0.1 per cent, used to protect blood against acid changes.

Changes in the Level of Carbon Dioxide Absorption Curves and Their Relation to Lactic Acid.—The carbon dioxide absorption curves which represent the carbon dioxide-combining capacity of oxygenated blood at different tensions are always lower after exercise. The levels of the curves at 40 mm. tension before and after work are tabulated in Columns 4 and 5 of Table II. In different individuals the amount of change varies considerably. The greatest deviation represents a fall of 42 per cent below the resting level of the curve.

The great reduction in bicarbonate and the fall in the level of the absorption curve has been attributed by many observers to the accumulation of lactic acid in the blood. Douglas and Haldane (26), studying the composition of alveolar air before and after exercise, predicted such a change in blood and considered

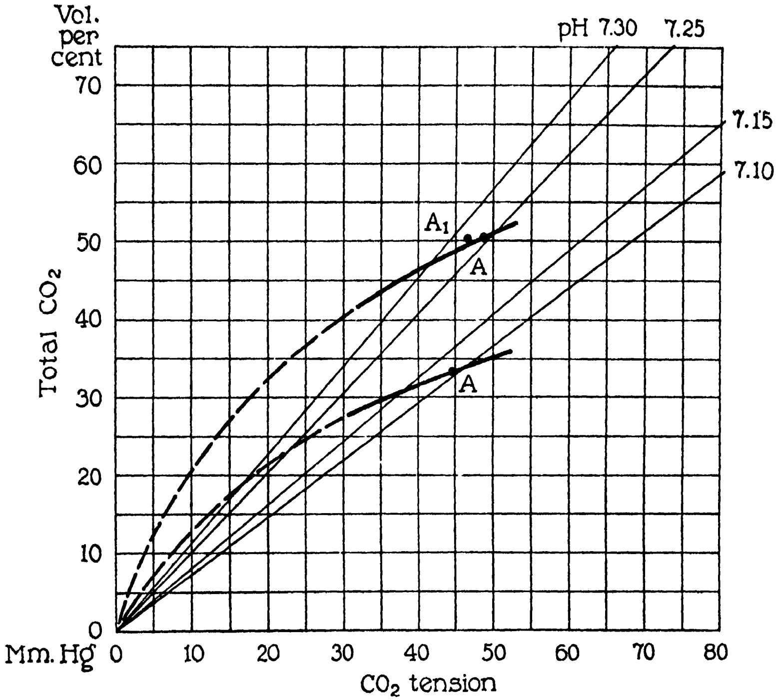

FIG. 1. Absorption curves before and after exercise. Experiment M. L Apr. 7, 1922. Ordinates represent the total carbon dioxide content of blood in volumes per cent. Abscissæ represent carbon dioxide tension in millimeters of mercury. Solid portion of curved lines indicate the part of absorption curve experimentally determined. Upper curve normal before exercise. Lower curve 3 minutes after exercise. The diagonal lines represent calculated pH values. Solid black circles indicate conditions of arterial blood as drawn from the body. Uncorrected values are marked A. Those corrected for oxygen unsaturation A_1.

it to be due to lactic acid arising from insufficient oxygen supply to the muscles during violent exertion. The actual presence of increased amounts of acid in the blood during the period immediately following exercise was first demonstrated by Ryffel

(27). Some doubt was thrown upon the relationship of lactic acid to the decreased bicarbonate by Haggard and Henderson (28) who injected enormous amounts of lactic acid into dogs with surprisingly small changes in the bicarbonate of the blood. On the basis of these experiments, they state as their belief that such a condition as lactic acid acidosis never exists during life. If the reaction between bicarbonate and lactic acid were complete, the addition of 10 mg. of lactic acid to 100 cc. of blood should cause a disappearance of bicarbonate corresponding to 2.5 volumes per cent of CO_2. Experiments by Evans (20) show that the addition of 10 mg. of the acid to blood *in vitro* causes a change of 1.5 volumes per cent. Results similar, but not quantitatively identical, were obtained by Mellanby and Thomas (29) who suggest that lactic acid may combine with substances other than bicarbonates when added to blood.

In a few experiments, we have attempted to demonstrate the relation of bicarbonate and lactic acid *in vivo*. For the determination of the latter, we have used the method recently developed by Clausen (16). This was selected as the method most applicable to the small amounts of blood at our disposal. Clausen has pointed out, however, and it should be emphasized in this connection that the method, like its predecessors, is not specific for lactic acid, but may apply to other α-hydroxy-acids. All determinations were made in duplicate and the checks were satisfactorily close. The results of our experiments are given in Table III. In each case, the difference in the amount of lactic acid actually determined by analysis is compared with that calculated from the difference in CO_2 capacity assuming that the addition of 4 mg. of lactic acid should cause a fall of 1 volume per cent in capacity. The experiments clearly demonstrate that the fall in capacity is always accompanied by an increase in acid. When a definite relationship between the two is sought, however, the results are contradictory. In two instances the increase in acid was greater than was necessary to account for the change in capacity. In the others the increase in acid was not quite sufficient to account for the change in capacity.

Changes in Slope of Absorption Curves and Their Significance.—Not only are the curves lower after exercise but they also have a flatter slope; *i.e.*, for a given increase in CO_2 tension the extra

amount of CO_2 absorbed is less. Since the change in carbonic acid is always the same between any two tensions, the lessened absorption of CO_2 after exercise is due to a lessened formation of bicarbonate. The amount of the difference before and after exercise is represented in Table IV. In Columns 4 and 5 are given the amounts of CO_2 as bicarbonate at 40 and 50 mm., respectively. Columns 6 and 7 represent the differences in bicarbonate[2] between the two tensions before and after exercise. In any given individual the amount of bicarbonate formed with increasing tension is always greater before than after exercise, the average increase before exercise corresponding to 4.0 volumes per cent of CO_2, that after exercise to 3.3 volumes per cent. The flatter slope of the

TABLE III

Relationship of Changes in CO_2-Combining Capacity to Lactic Acid.

Subject.	Date.	CO_2 capacity at 40 mm.		Lactic acid.			Calculated difference.
		Before.	After.	Before.	After.	Difference.	
	1922	*vol. per cent*	*vol. per cent*	*mg.*	*mg.*	*mg.*	*mg.*
D. P. B.	Aug. 8	49.2	32.9	15.0	100.8	85.8	65.2
D. P. B.	" 15*	48.1	27.2	22.4	117.5	95.1	83.6
D. P. B.	Oct. 24*	46.4	35.4	24.4	64.8	40.4	44.0
H. E. H.	Aug. 10*	49.3	40.5	14.5	46.2	31.7	35.2
H. E. H.	" 25	47.9	39.3	14.0	46.6	32.6	34.4
M. F.	Oct. 19*	43.2	28.5	25.2	76.7	51.5	58.8

* Protocols in Papers II and III of this series.

curve after exercise demonstrates that the blood is a less efficient carrier of CO_2 at physiological tensions. For a given change in CO_2 pressure, it takes up less CO_2 in the tissues and liberates less in the lungs. It is interesting that Haggard and Henderson (14) observed a similar flattening in the low curves obtained after the injection of hydrochloric acid in dogs.

The reasons for the flatter slope of the absorption curves after exercise are not apparent. Van Slyke (30) and others have demonstrated that the absorption or dissociation of CO_2 depends

[2] The amount of CO_2 as carbonic acid was calculated by multiplying the tension of CO_2 in millimeters by the factor 0.0672. To obtain the CO_2 as bicarbonate, this value was subtracted from the total CO_2 absorbed.

TABLE IV.

Comparison of Slopes of Absorption Curves before and after Exercise.

Subject.	Date.	Blood sample taken.	CO₂ as bicarbonate. At 40 mm.	At 50 mm.	Difference before exercise.	Difference after exercise
	1921		*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
D. P. B.	Oct. 17	Before.	44.8	48.9	4.1	
	Dec. 13	After.	34.5	37.6		3.1
	" 23*	"	29.3	32.6		3.3
	1922					
	Mar. 31	"	27.7	31.0		3.3
	Apr. 17*	"	26.7	29.9		3.2
	" 10*	"	25.0	28.0		3.0
H. E. H.	" 14*	Before.	47.3	51.4	4.1	
	June 15*	"	47.2	50.7	3.5	
	Mar. 22	"	45.3	49.0	3.7	
	Apr. 21*	After.	42.4	45.8		3.4
	" 14*	"	38.4	41.7		3.3
	Mar. 29	"	38.1	41.0		2.9
	Apr. 21*	"	34.1	37.6		3.5
	" 5*	"	29.7	32.6		2.9
H. G. R.	" 12	Before.	44.1	48.3	4.2	
	" 27*	After.	39.9	43.5		3.6
	" 27*	"	35.0	38.3		3.3
M. L.	" 7	Before.	43.7	47.8	4.1	
	" 19*	After.	30.8	33.9		3.1
	" 7	"	27.5	30.5		3.0
K. G. H.	" 3	Before.	46.3	50.3	4.0	
	" 3	After.	28.0	31.1		3.1
	1921					
N. P. L.	Nov. 18	Before.	44.9	49.4	4.5	
	" 18	After.	32.0?	35.6?		3.6?
	1922					
	Apr. 24*	"	33.9	37.8		3.9
	" 24*	"	30.3	33.9		3.6
Average					4.0	3.3

* Protocols in Papers II and III of this series.

upon the loss or gain of alkali from buffer substances such as hemoglobin, phosphates, and serum proteins. We considered at first that hemoglobin, the most important of the blood buffers might act less efficiently as a CO_2 carrier at the lower levels of the absorption curve. This hypothesis is not tenable in light of the recent experiments of Van Slyke and his associates (31). To solutions of pure hemoglobin, they added varying amounts of NaOH. When the solutions were exposed to different tensions of CO_2, absorption curves at several levels were obtained. The slope of the curves was essentially the same at all levels. Hemoglobin acted as a CO_2 carrier with equal efficiency under the varying conditions. Apparently, the flattening of the curve after exercise cannot be explained by any difference in the behavior of hemoglobin. Changes in its concentration, however, might be of importance. This was evident in the experiments of Van Slyke. It was shown by Hasselbalch (32) and by Barr and Peters (33) in anemia that, in general, the slope of the absorption curve becomes flatter as the concentration of hemoglobin diminishes. This does not help, however, in the explanation of the slope of the curve after exercise. An increase in concentration of hemoglobin always accompanies and follows exercise, which should tend to make the curves steeper instead of flatter. Of the other buffers, it is known that any change in the concentration of phosphates would influence the slope of the curves. Embden and Grafe (34) have shown an increase in phosphate excretion in the urine following exercise but we have found no information concerning the concentration of phosphates in the blood under similar conditions. Too little is known concerning the behavior of the serum proteins to warrant discussion.

Carbon Dioxide Tension and Reaction of Blood.—The CO_2 tension in the blood after exercise depends upon the balance of several factors. During the exertion, the production of CO_2 has been enormously increased, principally by the great rise in metabolic activity, but also to some extent by the liberation of preformed CO_2 from the breakdown of bicarbonate in blood and tissues. The elimination of this great amount of extra CO_2 depends: (1) upon a sufficient increase in the rate of blood flow; and (2) upon the adequacy of pulmonary ventilation. Consider-

ing the magnitude and rapidity of changes during muscular exercise, it is not surprising to find a marked variation in the CO_2 tension of the blood in different individuals. The results are given in Columns 7, 8, and 9 of Table II. The tension is always lower after exercise, the amount of reduction varying from 1.3 to 13.5 mm.

In the preceding discussion it has been shown that both bicarbonate and carbonic acid are decreased in amount after exercise. The reaction of blood depends upon the value of the carbonic acid-bicarbonate ratio. Hence any variation from the normal reaction will be determined by the relative changes in the two components. In none of our experiments was the reduction in

TABLE V.

Changes in CO_2-Combining Capacity, CO_2 Tension, and Reaction of Venous Blood after Exercise.

Subject.	Date.	Amount of work.	CO_2 capacity at 40 mm.			CO_2 tension of venous blood.			pH		
			Before.	After.	Difference.	Before.	After.	Difference.	Before.	After.	Difference.
		kgm.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*mm. Hg*	*mm. Hg*	*mm. Hg*			
D. P. B.	Dec. 13, 1921	3,550	45.7	37.2	8.5	48.0	44.5	3.5	7.29	7.22	0.07
K. G. H.	Apr. 3, 1922	3,490	49.0	30.5	18.5	47.2	72.5	+25.3	7.33	7.03	0.30

carbonic acid sufficient to compensate for the change in bicarbonate. In all instances, the reaction of the blood was less alkaline after exercise. This is shown in Columns 10, 11, and 12 of Table II. The degree of reduction varied in the different individuals, the greatest changes being observed in the blood of P. R. in which the pH fell from 7.29 to 7.04.

Changes in Venous Blood.—Two experiments, similar to those described above, were performed on venous blood. In Table V, it will be seen that the changes in CO_2 capacity and in blood reaction are similar in direction to those noted in arterial blood. The greatest deviation in reaction so far observed after exercise was found in the blood of K. G. H., a reduction in alkalinity of

pH 0.30. The changes in CO_2 tension were not in the same direction in the two individuals. K. G. H. had a great increase after exercise while in D. P. B. the tension was slightly less than before.

Relation of Changes in Acid-Base Equilibrium to the Amount of Work.

With the vigorous exertion studied in the preceding section, there were great changes in CO_2 tension, CO_2 capacity, and reaction. It seemed desirable to determine how much work could be done without producing notable changes in the acid-base equilibrium. A number of experiments were done in which the subject, D. P. B., performed on different days, amounts of work varying from 1,020 to 4,530 kilogrammeters. The venous blood was studied before and after each period of exercise. The results appear in Table VI.

TABLE VI.

Effect of Increasing Amounts of Exercise on Venous Blood of a Normal Individual.

Date.	Amount of work.	CO_2 capacity at 40 mm. CO_2 tension.			pH		
		Before.	After	Difference.	Before.	After.	Difference.
1922	*kgm.*	*vol. per cent*	*vol. per cent*	*vol. per cent*			
Mar. 8	1,020	44.7	43.2	1.5	7.28	7.26	0.02
" 13	1,371	43.4	42.5	0 9	7.27	7.26	0.01
" 15	2,096	46.0	44.5	1.5	7.27	7.25	0.02
" 20	2,385	44.8	41.5	3.3	7.28	7.25	0.03
" 17	3,048	47.9	41.0	6.9	7.29	7.24	0.05
1921							
Dec. 13	3,550	45 7	37.1	8.6	7.29	7.22	0.07
1922							
Apr. 17	4,096		35.9			7.19	
Mar. 31	4,530	45.0	30.4	14.6	7.30	7.16	0.14

The rate of pedalling was kept approximately constant in all experiments, the amount of work being regulated by change in the weight lifted. The lighter tasks (from 1,020 to 3,048 kilo-

grammeters, inclusive) were done in 3 minutes. $3\frac{1}{2}$ minutes were allowed for the heavier work. In the first three experiments, the blood samples were taken 1 minute after exercise. In the fourth in which 2,400 kilogrammeters were done, blood samples taken at 1 and at 3 minutes showed practically identical values for pH and CO_2 capacity. With 3,000 kilogrammeters, the blood was taken at the same intervals. The sample 3 minutes

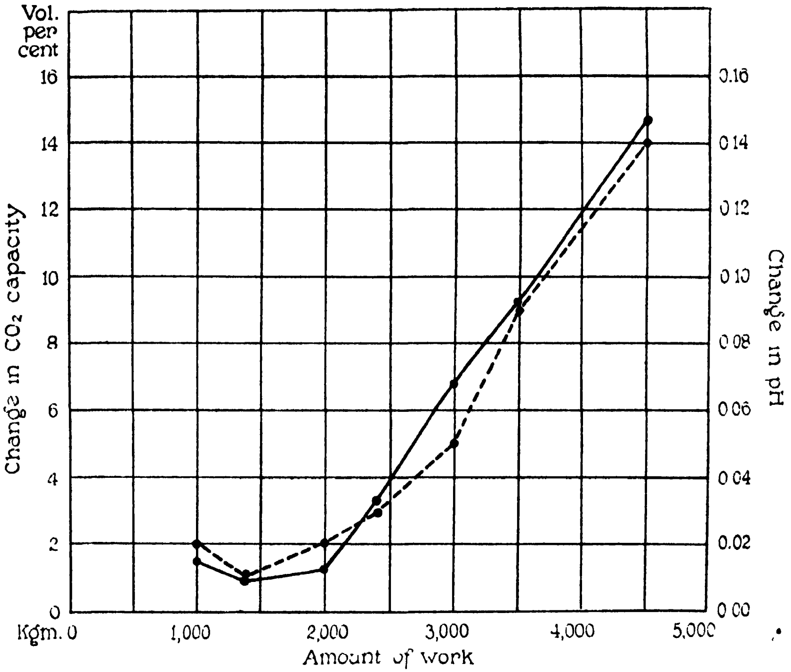

FIG. 2. Effects of increasing amounts of work upon CO_2-combining capacity and the reaction of venous blood. Solid line respresents change in CO_2 capacity. Broken line shows the change in pH.

after exercise, however, was not sufficient for the determination of CO_2 capacity. The CO_2 content indicated that the changes were greater in this sample. With the larger amounts of work the blood was drawn 3 minutes after exercise.

In Fig. 2, the changes in pH and CO_2 capacity are plotted against the amount of work, the solid line representing the CO_2 capacity, the broken line the pH. It will be noted that the

changes were just detectable when small amounts of work were done. With 1,020, 1,371, and 2,096 kilogrammeters the greatest change in pH was 0.02, in CO_2 capacity 1.5 volumes per cent. Even with 2,385 kilogrammeters the reaction of the blood was only slightly changed although the CO_2 capacity was reduced 3.5 volumes per cent. When more severe work was done, however, the degree of change increased with each increase in the task, until with 4,530 kilogrammeters, the pH was reduced 0.14 and the CO_2 capacity 14.6 volumes per cent. This represented the maximum work which the subject was able to do in the allotted time, greater exertion being impossible because of fatigue and pain in the calf muscles. Even with 4,530 kilogrammeters, the respiratory effort was not particularly distressing. The experiments demonstrate that a considerable amount of work (in this subject, about 2,000 kilogrammeters) can be done with scarcely detectable changes in the acid-base equilibrium of venous blood. With heavier work, the degree of change, both in reaction and CO_2 capacity of blood, increases approximately in direct proportion to the severity of the exertion. It should be mentioned, however, that the almost perfect parallelism between the changes in reaction and in CO_2 capacity is accidental. Such strict correspondence has not been found in other experiments nor should it be expected.

SUMMARY AND CONCLUSIONS.

1. Following short periods of vigorous muscular exercise, remarkable changes occur in the acid-base equilibrium of the blood. The CO_2-combining capacity is greatly diminished. The arterial CO_2 tension is reduced and the reaction of both arterial and venous blood becomes less alkaline. The degree of change varies considerably in different normal individuals.

2. The change in CO_2 capacity is accompanied by an increase in the concentration of lactic acid. The observed increase in lactic acid was compared with that calculated from the change in CO_2 capacity. No strict quantitative relationship could be demonstrated.

3. The slope of the CO_2 absorption curves is flatter after exercise. As a consequence, the efficiency of the blood as a CO_2

carrier is diminished. In the change between any two physiological tensions, a given volume of blood takes up less CO_2 from the tissues and eliminates less CO_2 in the lungs.

4. Easy muscular exercise may be performed with scarcely detectable changes in the acid-base equilibrium of venous blood. With heavier work the degree of change, both in reaction and in CO_2-combining capacity, increases rapidly with each small increase in the amount of work.

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Description of Subjects.

D. P. B.—Physician, age 32; weight, 76.4 kilos with clothes; height, 178 cm. with shoes; vital capacity, 4,500 cc. Takes no regular exercise. Smokes 15 to 20 cigarettes daily.

H. E. H.—Physician, age 28; weight, 68 kilos with clothes; height, 169 cm. with shoes; vital capacity, 4,300 cc. Takes no regular exercise. Does not use tobacco or alcohol.

N. P. L.—Physician, age 32; weight, about 77 kilos with clothes; height, about 170 cm. with shoes. Athletic individual with record at football and track. Takes constant exercise. Does not use tobacco or alcohol.

H. B. R.—Physician, age 33; weight, 70 kilos without clothes; height, 177 cm. without shoes; vital capacity, 4,300 cc. Takes no regular exercise. Smokes 10 to 15 cigarettes daily.

J. McL.—Medical student, age 25; weight, 68.2 kilos with clothes; height, 178 cm. with shoes; vital capacity, 4,900 cc. Has been an athlete, but takes no regular exercise at present. Does not use tobacco or alcohol.

M. L.—Medical student, age 25; weight, 71.8 kilos with clothes; height, 175 cm. with shoes; vital capacity, 4,400 cc. Athletic but takes no regular exercise at present. Smokes 15 to 20 cigarettes daily.

K. G. H.—Medical student, age 32; weight, 59.6 kilos with clothes; height, 175 cm. with shoes; vital capacity, 4,350 cc. Has been in poor physical condition for past 2 years following attack of encephalitis lethargica. Does not use tobacco or alcohol.

P. R. E.—Physician, age 27; weight, about 77 kilos with clothes; height, about 172 cm. with shoes; vital capacity, 3,700 cc. Lungs are slightly emphysematous. Expansion of chest limited. Takes no regular exercise. Does not smoke. Had never ridden a bicycle.

M. F.—Laboratory assistant, age 23; weight, 66 kilos with clothes; height, 166 cm. with shoes; vital capacity, 3,900 cc.; a former soldier in the British army in fair physical condition at present. Smokes 10 cigarettes daily.

J. E.—No occupation, age 28; weight, about 70 kilos with clothes; height, about 180 cm.; a fine physical specimen but a heavy drinker. Admitted to hospital following a spree of 3 weeks during which he drank cheap whiskey from many sources. Had a moderate degree of acidosis, but no eye symptoms. Reaction to exercise was studied 7 days after end of spree.

P. R.—A vagrant artist, age 26; weight, 68 kilos with hospital clothes; height, 170 cm. with shoes; healthy and robust in appearance. Admitted to hospital during an attack of acute tonsillitis. Reaction to exercise was studied 4 days after fever had subsided but before patient had completely regained his strength.

Protocols.

Subject.	Date.	Time blood was taken.	CO_2 absorption curve. CO_2 tension.	CO_2 absorption curve. CO_2 content.	CO_2 content of blood as drawn.	O_2 content of blood as drawn.	O_2 capacity.	Remarks.
	1931		*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
D. P. B.	Oct. 17	Before exercise.	21.2	35.0			23.2	Venous blood.
				35.1				
			78.0	62.8				
				63.4				
D. P. B.	Dec. 13	Before exercise.	39.5	46.1	54.1	11.7		Venous blood.
				44.9	53.3	11.2		Exercise = 3,550 kgm. in $3\frac{1}{2}$ min.
		3 min. after exercise.	24.0	28.4	40.9	14.3		
				29.6	41.2	14.2		
			39.9	37.4				
				37.0				
			52.1	41.6				
	1932							
D. P. B.	Mar. 8	Before exercise.	40.7	44.8	50.5	16.2		Venous blood.
				45.2	50.2	15.3		Exercise = 1,020 kgm. in 3 min.
		1 min. after exercise.	39.9	43.1	51.9	11.1		
				43.1				

D. P. B.	Mar. 13	Before exercise.	41.6	44.0	49.2	14.8		Venous blood.
				44 0	49 8	14.8		Exercise = 1,371 kgm. in 3 min.
		1 min. after exercise.	39.2	42 8	49.0	13.3		
				41.2	49.5	12.7		
D. P. B.	Mar. 15	Before exercise.	40.9	47.0	56.0	10.0		Venous blood.
				46.2	55.7	10 0		Exercise = 2,096 kgm. in 3 min.
		1 min. after exercise.	41.4	45.0	53.1	8.9		
				45.2	53.8	8.7		
D. P. B.	Mar. 17	Before exercise.	41.5	47.7	59.9	4 5	22 8	Venous blood.
				48.0			23.5	Exercise = 3,048 kgm. in 3 min.
		1 min. after exercise.	41.5	41.6	55.2	6.0		
				41.9	55.9	6.3		
		3 min. after exercise.			51.7	7.3		
					51.7	8.0		
D. P. B.	Mar. 20	Before exercise.	40.0	45.3	53.7	8.4		Venous blood.
				44.2	54.4	8.2		Exercise = 2,385 kgm. in 3 min.
		1 min. after exercise.	39.9	41.4	46.8	14.8		
				41.5	47.0	14.7		
		3 min. after exercise.	40.4	41.5	51.3	7.6		
				40.7	51.9	7.1		

Protocols—Continued.

Subject.	Date.	Time blood was taken.	CO_2 absorption curve. CO_2 tension	CO_2 absorption curve. CO_2 content.	CO_2 content of blood as drawn.	O_2 content of blood as drawn.	O_2 capacity.	Remarks.
	1922		*mm. Hg*	*vol. per cent*	*vol per cent*	*vol. per cent*	*vol. per cent*	
D. P. B.	Mar. 31	Before exercise.	39.9	44.4	52.6	10.9		Venous blood.
				45.5	52.0	10.9		Exercise = 4,530 kgm. in 3½ min.
		1 min. after exercise.	40.5	37 6	47.2	5.8		
				36.7				
		3 min. after exercise.	39.9	30.5	37 6	8 5		
				30.2	37.0	8.8		
			61 1	37.6				
				38.6				
D. P. B.	Aug. 8	Before exercise.	38.4	48.2	47.5	19.7	20.5	Arterial blood.
				48.8			20.4	Exercise = 3,770 kgm. in 3½ min.
								Lactic acid:
		3 min. after exercise.	36.7	31.2	24.7	21.4	21.9	Before exercise......15.4, 14.7 mg.
				31.7			21.8	After exercise......109.9, 91.7 mg.

H. E. H.	Mar. 22	Before exercise.	22 3	39 1	50 3	20 0	21 3	Arterial blood.
				39 1	50 6	19 9		
			61 0	56 9				
				56 8				
H. E. H.	Mar. 29	Before exercise.	39 9	48 3	51 1	20 3		Arterial blood.
				48 4	51 5	19 8		Exercise = 3,055 kgm. in 3½ min.
		3 min. after exercise.	19 7	31 0	41 6	20 7		
				30 1	40 8	21 0		
			39 3	40 2				
				40 9				
			50 0	44 4				
H. E. H.	Apr. 5	Before exercise.	39 0	49 5	54 2	19 4		Arterial blood.
				49 9	54 8	19 7		Exercise = 3,954 kgm. in 3½ min.
		3 min. after exercise.	36 4	31 0	31 7	21 4		
				30 9	31 8	20 7		
			57 3?	38 4				
				38 6				

Protocols—Continued.

Subject.	Date.	Time blood was taken.	CO_2 absorption curve.		CO_2 content of blood as drawn.	O_2 content of blood as drawn.	O_2 capacity.	Remarks.
			CO_2 tension.	CO_2 content.				
	1922		*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
H. E. H.	Aug. 25	Before exercise.	39.1	47.3	50.4	17.9	17.4?	Arterial blood.
				47.6	51.4	17.2	17.5?	Exercise = 3,545 kgm. in 3½ min.
		3 min. after exercise.	38.3	38.1	41.4	18.2	18.4	
				39.1	40.9	17.8	19.3	
	1921							
J. E.	Oct. 26	Before exercise.	19.2	28.0	40.8	22.5	24.1	Arterial blood.
				29.1	40.7	21.9		
				30.5				
			36.8	40.0				
				40.0				
			67.9	53.2				
				52.5				
J. E.	Nov. 2	Before exercise.	39.0	42.6	45.0	20.3		Arterial blood.
				42.0	44.9			Exercise = 3,700 kgm. in 3½ min.
		3 min. after exercise.	39.5	28.0	24.1	22.5		Only one point obtained in CO_2 absorption curve after exercise. CO_2 content plotted on a curve of average slope passing through this point.
				27.5	23.4			

N. P. L.	Nov. 15	Before exercise.	38.9	45.9	48.2	23.0	24.5	Arterial blood before and 3 min. after exercise. Venous blood 11 min. after.
				45.2	48.2	23.1		
		3 min. after exercise.	21.7	25.5	34.9	23 9		Exercise = 3,595 kgm. in 3½ min.
				25.3	34.9	24.0		
			40.6	34.5				
				35.4				
		11 min. after exercise.	40 3	39.6				
				39.9				
N. P. L.	Nov. 18	Before exercise.	41.4	48 3				Venous blood taken ½ hr. after light lunch. All other experiments done in late hours of morning.
				48.5				
			57.5	56.9				
				56.2				
J. McL.	Nov. 30	Before exercise.	36 8	46.4	51.3	20.9	22.4	Arterial blood.
				47.6	50.5	21.2		Exercise = 3,605 kgm. in 3½ min.
			48.9	51.7				Subject felt faint after first blood was drawn.
				52.1				
		3 min. after exercise.	22.8	27.3	32.3	22.2		
				26.9	32.3	22.6		
			34.2	30.9				
				30.9				

Protocols—Concluded.

Subject.	Date.	Time blood was taken.	CO_2 absorption curve.		CO_2 content of blood as drawn.	O_2 content of blood as drawn.	O_2 capacity.	Remarks.
			CO_2 tension.	CO_2 content.				
	1922		*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
		8 min. after exercise.	36 6	41.1	41.3	22.1		
				41 0	41.6			
P. R.		Before exercise.	40 5	45.5	46 3	20.5	22.4	Arterial blood.
				44.7	46 0	19 8	22.0	Exercise = 3,695 kgm. in 3½ min.
		3 min. after exercise.	26.8	20.4	26.9	21.0		
				20.1	25.6	20.9		
			40.1	26.2	25.9	21.2		
			50.6	30.2				
				29.1				
K. G. H.		Before exercise.	40.6	48.6	57.5	10.0	23.4	Venous blood.
				50.0	57.2	10.0	23.3	Exercise = 3,490 kgm. in 3½ min.
			66.6	60.2				
				60.8				
		3 min. after exercise.	41.7	31.3	46.5	7.4		
				31.2	47.3	6.9		
			66.7	38.9				
				40.6				

M. L.	1922 Apr. 7	Before exercise.	31.7	41.9	50.9	21.0	23.6	Arterial blood.
				41.4	50.3	21.6	24.1	Exercise = 3,285 kgm. in 3½ min.
			53.2	52.2				
				53.0				
		3 min. after exercise.	30.6	27.7	33.1	22.5		
				27.7	33.4	22.2		
			44.4	32.7				
				33.7				
H. B. R.	Apr. 12	Before exercise.	39.3	46.3	47.9	20.9	22.5	Arterial blood before and after exercise.
				46.4	48.6	20.3	22.7	
			55.7	54.3				
		3 min. after exercise.	67.0	45.8	34.2	21.7		Exercise = approximately 3,500 kgm. in 3½ min. Exact measurement lost. See protocols of other experiments of H. B. R. in Paper II.*
				46.3	33.9	21.2		

* Barr, D. P., and Himwich, H. E., *J. Biol. Chem.*, 1923, lv, 525.

STUDIES IN THE PHYSIOLOGY OF MUSCULAR EXERCISE.

II. COMPARISON OF ARTERIAL AND VENOUS BLOOD FOLLOWING VIGOROUS EXERCISE.

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In normal resting individuals, Parsons (1) found by calculation from electrometric determinations that the reaction of arterial and venous blood from the arm differed only by pH 0.02, the venous being less alkaline. Peters, Barr, and Rule (2) in three normal men calculated differences of only pH 0.01 to 0.00. No comparisons of the CO_2-combining capacity of whole blood from artery and vein have been found in the literature. Stadie and Van Slyke (3) compared the CO_2 capacity of arterial and venous plasma and showed that the venous was usually about 15 per cent higher. Joffe and Poulton (4) have demonstrated, however, that values for plasma CO_2 capacity depend upon the tension under which blood previously existed in the body. Since the CO_2 tension of venous blood is always higher than that of arterial, one should expect the capacity of venous plasma to be higher. Capacity determinations upon whole blood do not depend upon preexisting tensions and the results of Stadie and Van Slyke are, therefore, not applicable to whole blood studies. It has been generally assumed that the CO_2 capacity of arterial and venous blood is normally identical.

In the experiments of the preceding paper, it was demonstrated that the reaction and CO_2 capacity of both arterial and venous blood were diminished after exercise. The degree to which each was changed in any particular experiment, however, was not

determined. Consequently, it seemed desirable to compare the changes of arterial and venous blood drawn simultaneously after exercise. The procedure and methods of investigation are given in the first paper of this series.

In the experiments, one observer punctured the brachial artery. As soon as the needle has been introduced, a second observer punctured a vein of the other arm. As the venous blood began to flow, the volume was called out, cubic centimeter by cubic centimeter, until the required amount had been obtained. The rate of withdrawal of the arterial blood was regulated to

TABLE I.

Comparison of Arterial and Venous Blood after Exercise of Leg Muscles.

Subject.	Date.	Amount of work.	CO_2 capacity at 40 mm. tension.			pH		
			Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.
	1922	*kgm.*	*vol. per cent*	*vol. per cent*	*vol. per cent*			
H. B. R.	Apr. 12	3,500?	35.7	39.7	+4.0	7.22	7.20	−0.02
	" 27	3,846	37.8	42 8	+5.0	7.28	7.28	±0.00
D. P. B.	" 17*	4,092	29.5	35.9	+6.4	7.13?	7.19?	+0.06?
	June 20	3,855	32.2	34.9	+2.7	7.22	7.22	±0.00
	Aug. 15	3,820	27.2	32.7	+5.5	7.13	7.19	+0.06
M. L.	Apr. 19	3,655	30.2	33.5	+3.3	7.12	7.12	±0.00
N. P. L.	" 24	4,278	33.0	36.7	+3.7	7.17	7.16	−0.01
P. R. E.	June 17†	3,100	32.6	33.2	+0.6	7.19	7.15	−0.04
H. E. H.	Oct. 11	3,700	34.7	39.7	+5.0	7.21	7.21	±0.00

* Venous sample slightly exposed to air.

† Subject unaccustomed to bicycle riding. Considerable exercise with arms.

correspond to that of the venous. The technique was not easy and several samples had to be discarded because the flow from the two vessels could not be satisfactorily regulated. The results of nine experiments upon six individuals are given in Table I.

CO_2-Combining Capacity in Arterial and Venous Blood and the Rôle of Lactic Acid.

The original object in undertaking these experiments was to determine the relative values for pH in arterial and venous blood

after exercise. It was erroneously assumed that the CO_2-combining capacity would be the same in both samples. The fallacy of this was demonstrated in the first experiment (H. B. R., April 12) in which there was not enough of either arterial or venous blood to allow of the determination of the CO_2 absorption curve at three tensions. Two samples of venous blood were exposed to low CO_2 tensions while a specimen of arterial blood was used for exposure to a high tension. The resulting absorption curve was much flatter in slope than any that had been obtained in our previous work. Furthermore, when the arterial content was plotted on this curve, an alkalosis was indicated. On the other hand, the venous content when placed on the curve gave evidence of a marked acidosis. The apparent difference between arterial and venous reaction was pH 0.35. Although the technique of the experiment was entirely satisfactory and duplicate analyses were in excellent agreement, the results seemed impossible. The experiment was repeated. In the second observation, separate absorption curves were constructed for arterial and for venous blood. To our surprise, it was found that the curve obtained from arterial blood was 5 volumes per cent lower than that of the venous blood. When the contents of arterial and venous blood were plotted on their respective curves, the results were comparable to our previous findings. Both showed evidence of a considerable acidosis. The relations are illustrated in Fig. 1.

With the information which was obtained in the second observation it was possible to demonstrate the conditions which existed in the first. The capacity of venous blood had been experimentally determined at two tensions; that of arterial at a single tension. From these points curves were constructed using the slope experimentally determined in the second observation. It was found that the absorption curve of arterial blood was 4 volumes per cent lower than that of the venous. In seven similar experiments on five other subjects, the CO_2-combining capacity of arterial blood was found to be less than that of venous. The difference was large (from 2.7 to 6.4 volumes per cent) in all but one observation. In this (P. R. E., June 17) the difference was only 0.5 volume per cent which was within the experimental error of the method. The subject had never ridden a bicycle before and it was only with the greatest difficulty that

he completed 3,100 kilogrammeters of work in 3½ minutes. While other subjects during the ride simply supported themselves on the handle bars, P. R. E. used his arms vigorously as he swayed his body from side to side. The exercise of arm muscles undoubtedly altered the conditions of the experiment and may explain the smaller difference between arterial and venous blood.

An explanation of the unexpected difference in CO_2-combining capacity was sought. The increased capacity of venous blood

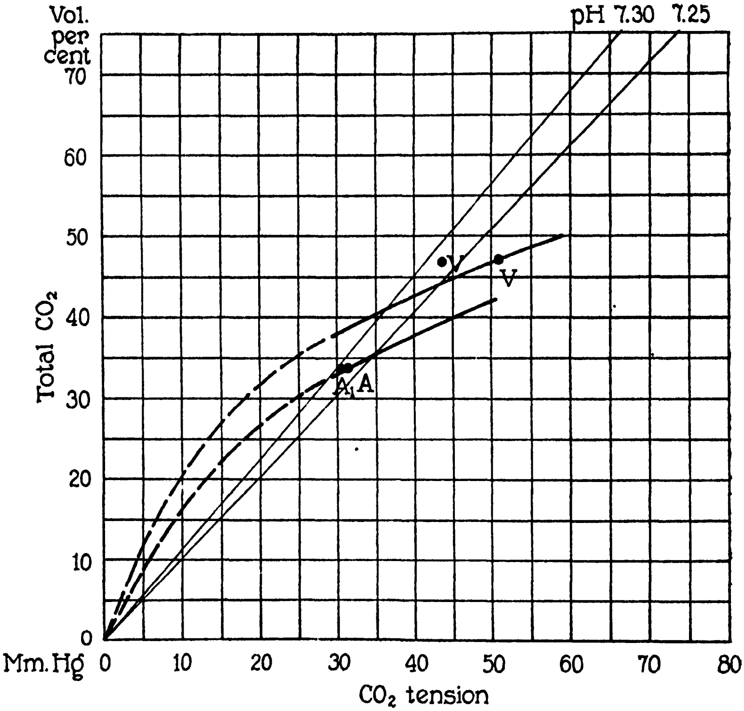

FIG. 1. Absorption curves of arterial and venous blood 3 minutes after exercise. Experiment H. B. R., Apr. 12, 1922. Ordinates represent the total carbon dioxide content of blood in volumes per cent. Abscissæ represent carbon dioxide tension in millimeters of mercury. Solid portion of curved lines indicates the part of absorption curve experimentally determined. Upper curve is of venous blood. Lower curve of arterial blood. Diagonal lines represent calculated pH values. Solid black circles indicate conditions of arterial and venous blood as drawn from the body. Uncorrected values are marked A and V. Those corrected for oxygen unsaturation A_1 and V_1.

might conceivably be due to a gain in alkali or to a loss of acid during the passage of the blood through the tissues of the arm. Concerning the former possibility we can find no direct evidence. The latter receives some support from Ryffel's (5) experiments. He found immediately after and as a result of exercise that the amount of lactic acid was greatly increased in the blood. An hour later the content of lactic acid had returned to its former resting level. That in this interval the acid was removed either directly or by oxidation is obvious. It seems possible from this evidence that the difference in capacity between arterial and venous blood which we have observed might represent a stage in the process of removal of lactic acid after exercise. The very magnitude of the difference, however, causes one to doubt the explanation. An increase of 5 volumes per cent in CO_2-combining capacity indicates the removal of at least 20 mg. of lactic acid from each 100 cc. of blood passing through the arms. If the process occurred at this rate in all tissues, one might expect the lactic acid, which was formed by the exercise, to disappear completely in a few minutes. On the other hand, the persistence of a low CO_2 capacity 15 to 30 minutes following exercise indicates that the removal is by no means so rapid. (See Paper III of this series.)

The question was submitted to experiment in two instances given in Table II. Both CO_2 capacity and lactic acid were determined in arterial and venous blood taken simultaneously after exercise. The lactic acid values were obtained by the recently published method of Clausen (6). In the last two columns of Table II, the observed difference in lactic acid is compared with that calculated from the change in CO_2 capacity on the basis that 4 mg. of lactic acid are the chemical equivalent of 1 volume per cent of CO_2. The satisfactorily close agreement between the two indicates that the removal of lactic acid by the tissues offers the true explanation for the differences in CO_2 capacity in arterial and venous blood.

In all of the observations except that of P. R. E., the active exercise was limited to the muscles of the legs. The arms were used only to support the weight during vigorous pedalling. Three other experiments were done in which exercise was performed entirely with the muscles of the forearm. The subject rested his

arm upon a table and lifted with his fingers and hand a weight of 1.5 kilos to the point of subjective fatigue. Samples of blood were drawn simultaneously from the brachial artery and median cephalic vein of the exercising arm after the cessation of work. The results are given in Table III.

The individual experiments are not comparable because the rate of lifting was not accurately standardized, the time consumed

TABLE II.

Relation of Lactic Acid to CO_2 Capacity in Arterial and Venous Blood.

Subject.	Date.	Source of blood.	CO_2 capacity at 40 mm.	Lactic acid content.	Observed difference in lactic acid.	Calculated difference in lactic acid.
	1922		*vol. per cent*	*mg.*	*mg.*	*mg.*
D. P. B.	Aug. 15	Artery.	27.2	118		
		Vein.	32.7	89	29	22
H. E. H.	Oct. 11	Artery.	34.7	80		
		Vein.	39.7	57	23	20

TABLE III

Comparison of Arterial and Venous Blood after Exercise of Arm Muscles.

Subject.	Date.	Source of blood.	CO_2 capacity at 40 mm.	Lactic acid content.	Observed difference in lactic acid.	Calculated difference in lactic acid.	pH
	1922		*vol. per cent*	*mg.*	*mg.*	*mg.*	
M. F.	Aug. 17	Artery.	41.6	20			7.20
		Vein.	41.6	47	27	0	7.20
M. F.	Aug. 22	Artery.	44.8	20			7.28
		Vein.	40.0	27	7	19	7.19
	Oct. 13	Artery.	38.5	17			7.16
		Vein.	38.5	21	4	0	7.16

in the work differed and the time at which the blood was taken after exercise varied (see protocols). Moreover, they are of necessity somewhat unsatisfactory since it is not certain how much of the blood in the superficial veins came from the exercised muscles. In all of the observations, however, the venous blood contains more lactic acid than does the arterial, a result exactly opposed to that obtained in similar samples after exercise of the legs. Also, the venous blood fails to show the increase in CO_2

capacity which was observed in the preceding experiments. In one observation, it is decreased 4.8 volumes per cent. In the others, it agrees with the arterial. There is, however, an entire lack of correspondence between the changes in CO_2 capacity and lactic acid. The reasons for this are not apparent.

In actively contracting muscles, lactic acid is produced and a portion of it escapes into the circulating blood. The experiments upon exercise of the arm, while not entirely satisfactory nor conclusive, demonstrate one fact clearly. The passage of lactic acid into the blood is still occurring actively from 2 to 4 minutes after the exercise has ceased. Simultaneously, as is shown by the leg exercise experiments, lactic acid is being rapidly removed during the passage of the blood through the inactive tissues of the arm. The amount of lactic acid and presumably the CO_2 capacity of arterial blood at any given time after exercise must depend upon the relative rates of production and removal of lactic acid in the tissues.

Reaction of Arterial and Venous Blood.

In six out of nine experiments with leg exercise (see Table I) the reaction of arterial and venous blood was almost identical, the difference being from pH 0.00 to 0.02. Two observations on D. P. B. exhibited a reaction of venous blood pH 0.06 more alkaline than arterial. The first of these was uncertain because a few bubbles of air were accidentally drawn through the venous sample. In the other, however, the technique was excellent and there appeared no reason to doubt the accuracy of the result. In the experiment on P. R. E. in which there was active exercise of arm muscles during the cycling, the reaction of venous blood was pH 0.04 less alkaline than arterial. In one of the arm exercise experiments a similar result was obtained, the venous sample being pH 0.09 less alkaline. The reaction of arterial and venous blood was the same in the other two arm exercise observations.

The agreement between the reaction of arterial and venous blood is surprisingly close in most of the experiments. From the preceding discussion, however, it would seem that this agreement can be no more than accidental. Theoretically, the venous blood may, according to conditions, be more or less alkaline

than the arterial. When an individual exercises vigorously, the reaction of the blood becomes rapidly less alkaline. Under these circumstances one should expect the venous blood returning from the exercising muscles to be much less alkaline than the arterial blood which supplied them. Similarly, after exercise, the reaction of the blood gradually returns to normal. This implies that at some time following exertion, the venous blood returning from the tissues will be more alkaline than the arterial. The two results on D. P. B. and the single observation on P. R. E. thus become understandable.

Some of the factors which lead to variations in reaction between arterial and venous blood after exercise may be specified more definitely. CO_2 production and lactic acid formation in the tissues tend to make the blood less alkaline. The loss of oxygen from the blood and the removal of lactic acid tend to increase the alkalinity. Under resting conditions, it is probable that the acid effect of CO_2 production is largely neutralized by the alkaline effect of removal of oxygen so that the venous blood is only slightly less alkaline than the arterial. If this be true after exercise, it would appear that the difference in reaction should depend upon the production and removal of lactic acid. In P. R. E. and in the experiments with arm exercise the production of lactic acid in the tissues may have caused the venous blood to be less alkaline. In those instances in which the removal of lactic acid by the tissues was rapid, the venous blood might become more alkaline as in the experiments upon D. P. B. These factors do not explain the results of the other experiments in which the differences in lactic acid were quite as great but in which the reaction of arterial and venous blood was identical. A larger number of observations might have yielded greater variations.

SUMMARY AND CONCLUSIONS.

1. A comparison has been made of arterial and venous blood drawn simultaneously from the arm 3 minutes after vigorous exercise with leg muscles.

2. The CO_2-combining capacity is always higher in the venous blood, the increase varying from 0.6 to 6.4 volumes per cent and averaging 4 volumes per cent in nine experiments.

3. The higher CO_2 capacity of venous blood may be accounted for by a decrease in lactic acid content which is due to the removal of lactic acid from the circulating blood during its passage through the tissues of the arm.

4. A further comparison of simultaneous samples of arterial and venous blood from the arm has been made after vigorous exercise with the muscles of the forearm. Under these circumstances, the CO_2-combining capacity of the venous blood is the same as, or less than, the arterial capacity. The lactic acid content is greater in the venous blood.

5. The experiments indicate that 3 minutes after exercise, lactic acid is still escaping into the blood as it returns from exercising muscles. At the same time, the less active tissues are removing it rapidly as the blood passes through them. The lactic acid content and presumably the CO_2 capacity, which are found in arterial and venous blood at any instant after exercise, depend upon the relative rates at which lactic acid is produced and removed.

6. Surprisingly close agreement was found between the reaction of arterial and venous blood in eight out of eleven experiments. In two instances the venous blood was less alkaline; in two it was more alkaline.

7. The great difference in CO_2 capacity between arterial and venous blood makes it impossible to draw any conclusions concerning arterial conditions from a study of venous samples after exercise.

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Protocols.

Subject.	Date.	Source of blood specimen.	CO_2 absorption curve.		Lactic acid content.	CO_2 content of blood as drawn.	O_2 content of blood as drawn.	O_2 capacity.	Remarks.
			CO_2 tension.	CO_2 content.					
	1923		*mm. Hg*	*vol. per cent*	*mg.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
H. B. R.	Apr. 12	Artery.	67.0	45 8		34.2	21.7	22.5	Simultaneous arterial and venous blood 3 min. after exercise.
				46 3		33.9	21.2	22.7	Exercise = approximately 3,500 kgm. in 3½ min.
		Vein.	26.4	33.3		48.3	12.7		
				33.9		48.7	12.9		
			30.6	35.7					
				35.9					
H. B. R.	Apr. 27	Artery.	29.3	32 4		33.5	21.7		Simultaneous arterial and venous blood 3 min. after exercise.
				33 0		33.9	21.3		Exercise = 3,846 kgm. in 3½ min.
			50 3	42 0					
				42.2					
		Vein.	29.3	37.3		47.1	14.3		
				37.8		46.9	14.5		
			59.2	50.1					
				49.9					
D. P. B.	Apr. 17	Vein.	39.6	35.9		44.7?	8.6?		Simultaneous arterial and venous blood 3 min. after exercise.
				35.4		43.9?	9.4?		Exercise = 4,092 kgm. in 3½ min.
		Artery.	20.6	19.8		27.4	22.5		Venous blood exposed to negative pressure and air before analyses for content were made.
				20.2		27.0	22.5		
			39.1	29.1					
				29.0					
			72.9	40.0					
				40.1					

D. P. B.	June 20	Vein.	33.7	31.3		38.7	13.4		Simultaneous arterial and venous blood 3 min. after exercise.
				31.9					
			62.2	43.8					Exercise = 3,855 kgm. in 3½ min.
		Artery.	32.2	28.5		25.0	23.2		Blood protected against acid changes with 0.1 per cent sodium fluoride and low temperature.
				28.3		25.4	22.5		
D. P. B.	Aug. 15	Vein.	39.5	32.3	91.6	35.9	13.4	22.6?	Simultaneous arterial and venous blood 3 min. after exercise.
				32.3	86.1	36.2	13.6		
		Artery.	38.3	26.3	119.0	22.7	22.4	23.9	Exercise = 3,820 kgm. in 3½ min.
					116.0	22.6	22.9	23.7	Blood protected against acid changes with 0.1 per cent sodium fluoride.
M. L.	Apr. 19	Vein.	31.2	30.0		38.5	20.5		Simultaneous arterial and venous blood 3 min. after exercise. Arterial and venous blood did not flow at exactly the same rate.
				29.8		38.5	19.8		
			67.5	43.3					
				43.3					
		Artery.	30.4	26.4		29.7	22.0		Exercise = 3,655 kgm. in 3½ min.
				25.9		29.6	22.2		
			52.1	35.2					
				34.6					
N. P. L.	Apr. 24	Artery.	31.3	28.9		31.2	23.8		Simultaneous arterial and venous blood 3 min. after exercise.
				28.7		30.8	23.9		
			54.0	39.1					Exercise = 4,278 kgm. in 3½ min.
				38.9					
		Vein.	33.0	33.2		49.2	11.2		
				33.2		50.2	11.5		
			71.0	49.2					
				49.2					

Protocols—Concluded.

Subject.	Date.	Source of blood specimen.	CO_2 absorption curve.		Lactic acid content.	CO_2 content of blood as drawn.	O_2 content of blood as drawn.	O_2 capacity.	Remarks.
			CO_2 tension	CO_2 content.					
	1923		*mm. Hg*	*vol. per cent*	*mg.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
P. R. E.	June 17	Artery.	35 6	30 1		28.9	25.4	24.8	Simultaneous arterial and venous blood 3 min. after exercise.
				30.6		29 0	24 8		
		Vein.	34.4	31.0		44 2	8 8		Exercise = 3,100 kgm. in 3½ min.
				30 2		44 2	8.3		Subject, an inexperienced bicycle rider.
			57.4	40.0					Performed work with great difficulty and with much extra movement of body and arms. Blood protected with 0.1 per cent sodium fluoride and low temperature.
				39.3					
H. E. H.	Oct. 11	Artery.	38.6	34.2	80.5	33.2	21.4	21 3	Simultaneous arterial and venous blood 3 min. after exercise.
				33.6	78.4	32.6	21.1	20 7	
		Vein.	40.0	39.7	58.1	50.5	9.6	21.3	Exercise = 3,700 kgm. in 3½ min. Used sodium fluoride 0.1 per cent.
				39.7	55.3	49.8	8 6	21.6	
M. F.	Aug. 17	Artery.	36 4	40.0	20.3	47.1	22.1		Simultaneous arterial and venous blood 2 min. after exercise.
					18.9	47.0	22.1		
			37 3	40.2	46.2	50.2	17.5		Exercise = approximately 30 kgm. in 2 min. Used sodium fluoride 0.1 per cent.
					48 3	50.2	17 5		
			55.1	48.3					
				47.8					

M. F.	Aug. 22	Artery.	40 0	44 3	21 0	48 2	19 8	23 6	Simultaneous arterial and venous blood 3 min. after exercise.
				45 3	18 2	48 1	19 9		
		Vein.	40 0	41 1	27 3	49 5	14 8	23 6	Exercise = approximately 40 kgm. in 4 min. Used sodium fluoride 0.1 per cent.
				39 0	25 9	49 9	15 1		
M. F.	Oct. 13	Artery.	41 8	39 7	16 1	45 0	21 2	22 1	Simultaneous arterial and venous blood 4 min. after exercise.
				39 3	16 8	45 2	20 8	21 5	
		Vein.	41 9	39 4	22 4	54 2	10 1	21 9	Exercise = 50 kgm. in $3\frac{3}{4}$ min. Used sodium fluoride 0.1 per cent.
				39 7	20 3	53 5	9 9	21 2	

STUDIES IN THE PHYSIOLOGY OF MUSCULAR EXERCISE.

III. DEVELOPMENT AND DURATION OF CHANGES IN ACID-BASE EQUILIBRIUM.

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Three minutes after exercise, remarkable changes are found in the CO_2 tension and reaction of blood (1). Observations at this particular instant after exercise, however, tell us nothing of the changes which have occurred during the exercise nor do they inform us concerning the progress and rapidity of the recovery process.

The literature gives us little help with these questions. Douglas and Haldane (2) found that the CO_2 tension of alveolar air, obtained by the Haldane-Priestley (3) method, was increased immediately after severe exercise, diminished progressively for several minutes after exercise, and remained lower than normal for ½ hour or more after the work had stopped. Christiansen, Douglas, and Haldane (4) showed that the CO_2-combining capacity was greatly reduced 7 minutes after exercise, but had returned to its normal value in 77 minutes. Ryffel (5) showed that lactic acid was still increased in amount 45 minutes after a short period of vigorous exercise. So far as we know, the reaction and CO_2 tension of the arterial blood have not been studied either during exercise or at varying times after it.

It is known that the greatest increase in respiration as well as pulse rate and blood flow occurs during exertion. *A priori*, it might be expected that the greatest changes in acid-base equilibrium would also occur before the end of exercise. Haggard and Henderson (6), however, have suggested the hypothesis

that response to exercise is an acapnia phenomenon similar to that which they have observed experimentally in carbon monoxide poisoning and in individuals exposed to low oxygen pressures. The first reaction according to the acapnia theory is an overventilation of the lungs with a resulting alkalosis. This is followed by a compensatory reduction in the bicarbonate of the blood which according to the work of Macleod (7) may be in part accomplished by the production of lactic acid. It is conceivable, but not probable, that this compensatory process of bicarbonate reduction might proceed too far and cause an acidosis similar to that which we have found 3 minutes after exercise.

EXPERIMENTAL.

Experiments were done to determine the direction, development, and duration of changes which occur in the blood as a result of exercise. Complete protocols will be found at the end of the paper. The procedure and methods are described in the first article of this series (1).

Experiments on Venous Blood.—The first observation was made on the blood of D. P. B. (Dec. 23, 1921). Specimens were taken from an arm vein 1, 3, and 8 minutes after 4,332 kilogrammeters of work. The results were so contrary to anything we had expected that the experiment was repeated. In the second observation (D. P. B., Mar. 31, 1922) the blood was taken before exercise and 1 and 3 minutes after the work had ceased. Confirmation of the first results was obtained. To determine the duration of the changes and the stages in the recovery process, a third experiment was performed in which samples of blood were taken 8, 19, and 35 minutes after exercise (D. P. B., Apr. 10, 1922). Although the amount of work was approximately the same, the subject experienced much greater subjective fatigue. The degree of change in combining capacity and in reaction was also greater at the end of 8 minutes than it was in the first experiment. The results of the study on venous blood are given in Table I. A graphic representation of the first two experiments is given in Fig. 1, which is a modification of the CO_2 diagram introduced by Haggard and Henderson (6). In Fig. 1 the CO_2 absorption curves have been omitted in order that the reaction and CO_2 tension may be more clearly indicated.

Experiments on Arterial Blood.—The preceding observations were made on venous blood from the arm and could not be applied without reservations to general conditions in the body. A second set of experiments was performed on another individual, using arterial blood. In one observation (H. E. H., Apr. 14, 1922) blood was taken before and during the last minute of exercise. In a second (H. E. H., Apr. 5, 1922), samples were taken before and 1 and 3 minutes after, while in a third (H. E. H., Apr. 21,

TABLE I.

Direction and Duration of Changes in Venous Blood.

Exercise = approximately 4,400 kgm. in 3½ min.

Subject.	Date	Blood withdrawn	CO_2 capacity at 40 mm.		CO_2 tension of venous blood.		pH of venous blood	
			Capacity	Difference.	Tension	Difference	pH	Difference
			vol per cent	*vol per cent*	*mm.*	*mm.*		
D. P. B.	Mar. 31, 1922	Before exercise.	45.0		46.0		7.30	
		1 min. after exercise.	37.2	7.8	50.6	+4.6	7.22	0.08
		3 " " "	30.5	14.5	45.0	−1.0	7.15	0.15
D. P. B.	Dec. 23, 1921	1 " " "	37.2		53.2		7.21	
		3 " " "	32.0		46.5		7.14	
		8 " " "	32.0		40.8		7.18	
D. P. B.	Apr. 10, 1922	8 " " "	27.8		38.0		7.12	
		19 " " "	30.5		43.5		7.15	
		35 " " "	38.0		43.0		7.25	

1922), blood was drawn 3, 15, and 30 minutes after the exercise had ended. The amount of work was approximately 4,000 kilogrammeters in all experiments. Unfortunately, the reaction of the blood was not identical in the two experiments in which it was studied before exercise. For this reason a strict comparison of the reaction of the blood during and after exercise was not possible. The results of this group of experiments are given in Table II. The graphic representation of the second and third experiment is charted in Fig. 2.

Seven other experiments were performed in which the arterial blood was taken before and at varying intervals after the beginning of exercise. The duration and amount of work varied in the different observations. Results are included in Table II. In one experiment, arterial blood was secured during the 2nd minute of exercise, in three during the 3rd, and in another during the 7th. After exercise, blood samples were taken at intervals which varied from 3 to 49 minutes.

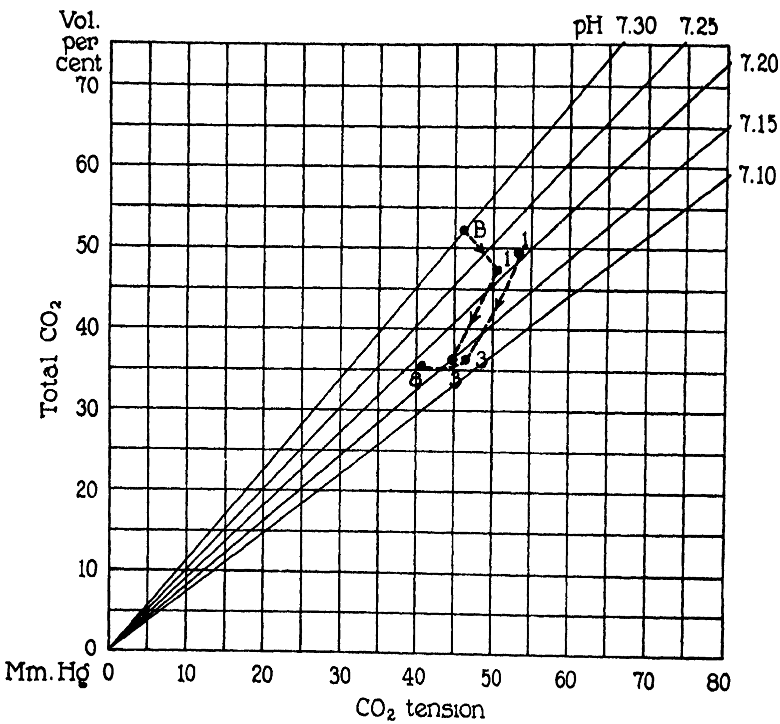

FIG. 1. Reaction, CO_2 content, and CO_2 tension of venous blood before and after 4,400 kilogrammeters of work. Ordinates represent the total carbon dioxide content of blood in volumes per cent. Abscissæ represent carbon dioxide tension in millimeters of mercury. Diagonal lines represent calculated pH values. Solid black circles indicate conditions of venous blood as drawn from the body. B represents conditions before exercise; 1, 3, and 8, the conditions 1, 3, and 8 minutes after cessation of work. CO_2 absorption curves are omitted in order that reaction and CO_2 tension may be more clearly indicated.

TABLE II.

Direction and Duration of Changes in Arterial Blood.

Subject.	Date.	Amount and duration of work.	Blood withdrawn.	CO_2 capacity at 40 mm.	CO_2 tension of arterial blood.	pH of arterial blood.
	1922			*vol. per cent*	*mm.*	
H. E. H.	Apr. 14	3,954 kgm. in 3½ min.	Before exercise.	50.1	40.0	7.35
			During last min. of exercise.	41.5	37.5	7.27
H. E. H.	" 5	4,098 " " 4 "	Before exercise.	50.1	48.0	7.30
			1 min. after exercise.	35.1	43.2	7.16
			3 " " "	32.5	38.5	7.15
H. E. H.	" 21	4,104 " " 3½ "	3 " " "	33.5	34.5	7.19
			15 " " "	37.0	36.5	7.23
			30 " " "	45.2	38.7	7.31
H. E. H.	Aug. 10	3,400 " " 3¾ "	Before exercise.	49.3	38.8	7.35
			During last min. of exercise.	42.9	34.5	7.32
			3 min. after exercise.	40 5	33.0	7.30
H. E. H.	" 25	3,545 " " 3⅓ "	Before exercise.	47.9	47.0	7.28
			During last min. of exercise.	40.2	48.0	7.20
			3 min. after exercise.	39.3	44.5	7.21
H. E. H.	Oct. 27	3,408 " " 3½ "	Before exercise.	47.0	48.0	7.26
			During 2nd min. of exercise.	44.2	52.2	7.21
D. P. B.	" 24	3,504 " " 3⅓ "	Before exercise.	46.4	39.7	7.31
			During last min. of exercise.	41.4		
			15 min. after exercise.	35 4	33.0	7.23
D. P. B.	June 21	7,380 " " 7½ "	Before exercise.	46.5	39.5	7.33
			During last min. of exercise.	29.5	29.5	7.16
			5 min. after exercise.	29.5	29.5	7.16
	1921					
J. McL.	Nov. 30	3,605 " " 3½ "	Before exercise.	48.4	45.2	7.30
			3 min. after exercise.	32.6	39.5	7.15
			8 " " "	42.5	36.6	7.30
J. E.	" 12	3,985 " " 3½ "	Before exercise.	44.6	39.2	7.30
			49 min. after exercise.	41.0	37.5	7.27

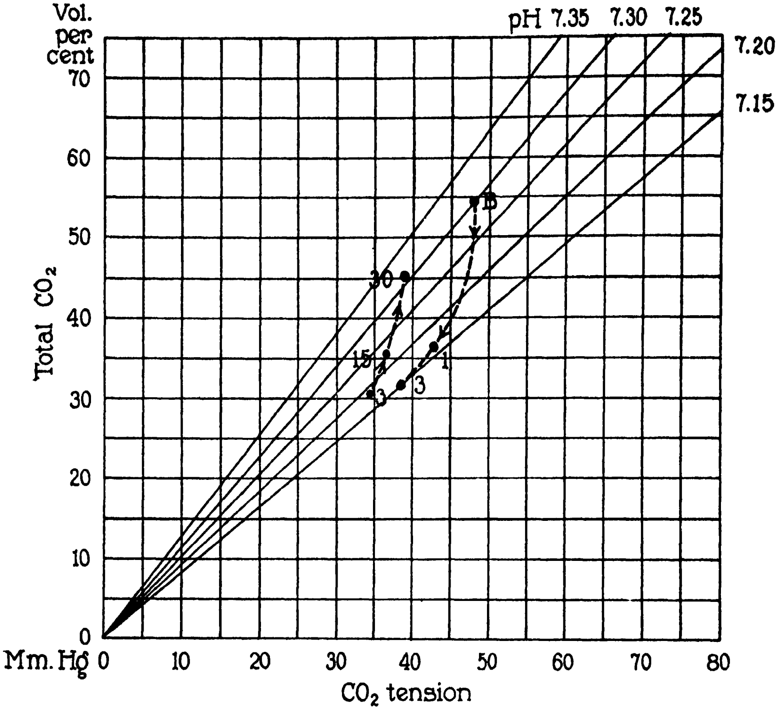

FIG. 2. Reaction, CO_2 content, and CO_2 tension of arterial blood before and after 4,000 kilogrammeters of work. Method of representation as in Fig. 1. B represents conditions before exercise; 1, 3, 15, and 30, conditions 1, 3, 15, and 30 minutes after the end of exercise.

DISCUSSION.

Changes in CO_2-Combining Capacity.

A diminution of 2.8 volumes per cent in the CO_2 capacity was apparent in the arterial blood after 2 minutes of work in which the rate was 973 kilogrammeters per minute (H. E. H., Oct. 27, 1922). In the same subject, after 3 minutes of exercise, the reduction in capacity amounted to 6.4 volumes per cent when the rate was 900 kilogrammeters, to 7.7 volumes per cent with a rate of 1,070 kilogrammeters, and to 9.3 volumes per cent with a rate of 1,130 kilogrammeters. When the duration of exercise was only 3½ minutes, the change in capacity did not reach a maximum

during exercise, but continued to increase for several minutes after the work had ended (H. E. H., Aug. 10, 1922 and Aug. 25, 1922). In one experiment (D. P. B., Nov. 25, 1922) it was greater 15 minutes after exercise than it was during the last minute of work. When the duration of work was 7½ minutes (D. P. B., June 21, 1922) the CO_2 capacity during the last minute of exercise was the same as it was 5 minutes later, indicating that a maximum change had occurred during the exertion. The return to normal conditions was well under way 15 minutes after the end of work, but was not complete after 30 minutes in H. E. H., (Apr. 21, 1922) nor after 49 minutes in J. E. (Nov. 12, 1921).

It was demonstrated by Barr and Himwich (8) that 3 minutes after exercise of leg muscles, the CO_2 capacity of venous blood of the arm was from 2 to 6 volumes per cent higher than the arterial. It was not likely, therefore, that the development and duration of changes would be the same in arterial and venous blood. Superficially, however, they were quite similar. In the venous as in the arterial blood the reduction in CO_2 capacity was progressive after the end of exercise. The change was greater at 3 minutes than it was at 1 minute. After 8 minutes it was the same as it was at 3 minutes. 19 minutes after exercise the return to normal conditions had commenced, but at 35 minutes it was far from complete.

The significance of the changes in CO_2 capacity may be best interpreted by a consideration of lactic acid formation. That lactic acid appears in large amounts in the circulating blood after exercise was first shown by Ryffel (5). The experiments of Barr, Himwich, and Green (1) indicated that lactic acid accumulation is the chief cause of the reduction in CO_2 capacity. Although the lactic acid is probably produced only during the active muscular work, it continues to escape into the circulation for some time after the exercise has ceased. Evidence of this appeared in the arm exercise experiments of Barr and Himwich (8) in which it was found that lactic acid is still accumulating in venous blood from 2 to 4 minutes after the cessation of work. Further evidence has been furnished by the results of the present experiments, in which after 3½ minutes of work, the CO_2 capacity is progressively lowered for several minutes after the exertion. The amount of lactic acid which enters the circulation after

$3\frac{1}{2}$ minutes of exercise must be large for at the very time in which it is seen to be accumulating, portions of it are being removed. The experiments of Barr and Himwich (8) with leg exercise have shown that 3 minutes after work, from 10 to 20 mg. are removed from each 100 cc. of blood in its passage through the tissues of the arm. Thus the recovery process which commences with the removal of lactic acid from the blood is far advanced 3 minutes after exercise. The CO_2 capacity continues to diminish, however, until the rate of removal of lactic acid equals the rate at which it escapes into the blood from exercising muscles. With $7\frac{1}{2}$ minutes of work, the CO_2 capacity did not diminish after exercise. This may signify that with work of longer duration, the rates of removal and accumulation of lactic acid become equal during the exertion.

CO_2 Tension.

The CO_2 tension of arterial blood was somewhat variable. During the 2nd minute of work it was increased 4.2 mm. in H. E. H. (Oct. 27, 1922). In the same subject, during the 3rd minute of exercise, it was increased 1.0 mm. in the experiment on August 25, 1922, and decreased 2.5 and 4.3 mm. in the experiments of April 14 and August 10, respectively. During the 7th minute of work in another subject (D. P. B., June 21, 1922) there was a great reduction in CO_2 tension, from 46.5 to 29.5 mm. After $3\frac{1}{2}$ minutes of exercise, the arterial tension fell for several minutes (H. E. H., Aug. 10 and 25, 1922). It increased slightly after 15 minutes, but it had not returned to normal in 30 minutes (H. E. H., Apr. 21, 1922). In the venous blood the tension remained high for a longer period. 3 minutes after exercise it was higher than normal. At 8 minutes it was less than normal and had not returned to the original resting value 35 minutes after exercise.

The results indicate that during the 2nd minute and in one instance during the 3rd minute of exercise CO_2 has accumulated in the blood. During these first few minutes, the respiration is not sufficient to remove CO_2 as fast as it is produced. If the work is continued, however, the respiration becomes competent not only to eliminate the great amount of CO_2 evolved by the exercise but also to pump out a considerable part of the preformed

CO_2. This is apparent in two experiments during the 3rd minute of work and is striking in the observation in which the duration of exercise was 7½ minutes.

Reaction of Blood.

A change in the reaction of arterial blood was apparent during the 2nd minute of exercise. In the experiment on H. E. H. (Oct. 27) the blood was pH 0.05 less alkaline during the exercise than it was during rest. In the three experiments in which blood was taken during the 3rd minute of work, the blood was also less alkaline, the deviation from normal varying between pH 0.03 and 0.08 (H. E. H., Apr. 14, 1922, Aug. 10, 1922, and Aug. 25, 1922). The change in reaction was much greater when 7½ minutes of work was performed, being pH 0.17 during the 7th minute of exercise (D. P. B., June 21, 1922). With 3½ minutes of exercise, the arterial blood was in two instances slightly less alkaline 3 minutes after exercise than during it. In another experiment it was pH 0.01 more alkaline 3 minutes after work. With 7½ minutes of exercise, the reaction during the last minute of exertion and 5 minutes later was identical. The return to normal varied in the different subjects. In H. E. H. (Apr. 21, 1922) the recovery had commenced after 15 minutes and was probably complete in 30. In J. McL. (Nov. 30, 1921) it had returned to normal in 8 minutes. In D. P. B. (Oct. 24, 1922) it was pH 0.08 less alkaline after 15 minutes while in J. E. (Nov. 12, 1921) the reaction was slightly less alkaline after 47 minutes. In the venous blood the reaction became progressively less alkaline during the first 3 minutes after exercise. Recovery had commenced in 8 minutes, but was not complete in 35.

The experiments demonstrate that the response to exercise is not an acapnia phenomenon. The theory of acapnia assumes a primary overventilation of the lungs with a decrease in CO_2 tension and an alkalosis. At the beginning of exercise, the pulmonary ventilation is inadequate to remove the CO_2 as fast as it is formed and there is an actual increase in CO_2 tension. There is no preliminary alkalosis. Even during the 2nd minute of exercise the reaction becomes less alkaline and the acidosis increases as the work is continued. According to the acapnia hypothesis, reduction in bicarbonate is a compensatory process

TABLE III.

Duration of Changes in CO_2 Tension, Lactic Acid Content, and Reaction of Arterial Blood during and after Exercise.

Time.	CO_2 tension.	Lactic acid content.	Alkalinity pH.
During 1st and 2nd min. of exercise	Increasing.	Increasing.	Diminishing.
" 3rd " 4th " " "	Decreasing.	"	"
From end of exercise to 3rd min. after	"	"	"
" 3rd to 8th min. after exercise	"	Decreasing.	Increasing.
" 8th " 15th " " "	Increasing.	"	"
" 15th " 30th " " "	"	"	" to normal.
" 30th " 50th " " "	" to normal.	" to normal.	Normal.

which follows the original fall in CO_2 tension. In exercise, reduction in bicarbonate occurs simultaneously with an increase in CO_2 tension.

With 3½ minutes of work the maximum change in reaction does not occur during the exercise. In several experiments, the blood became still less alkaline after the work had ended. The reasons for the long continued acidosis after exercise follow from the preceding discussion of CO_2 capacity, lactic acid content, and CO_2 tension. They may be more readily understood by a consideration of Table III which indicates the direction of changes in the CO_2 tension, lactic acid content, and reaction of arterial blood during and after the standard amount of exercise. Since the reaction of blood depends upon the carbonic acid-bicarbonate ratio, it will be influenced by any change either in CO_2 tension or in lactic acid content. Changes in tension increase or decrease carbonic acid while variations in lactic acid change the amount of bicarbonate. During the first 2 minutes of exercise, both CO_2 and lactic acid accumulate in the blood with a resulting acidosis. As the exercise continues CO_2 is removed by the increasing respiration until at the end of 3 to 4 minutes of exercise it becomes definitely less than normal. If this were the only factor the reaction would become more alkaline as the work continued. The simultaneous accumulation of lactic acid, however, prevents this and is so great that the reaction becomes progressively less alkaline during the exercise and in some cases for several minutes afterwards.

SUMMARY AND CONCLUSIONS.

1. CO_2 capacity, CO_2 tension, and reaction of arterial and venous blood were studied before and at various times during and after short periods of muscular work, in order to determine the direction, rate of development, and duration of the changes in acid-base equilibrium.

2. With 3½ minutes of work, the CO_2 capacity of arterial blood was diminished during the 2nd minute of exercise. It became progressively lower during the exertion and for several minutes (3 or more) after the exercise had ended. The return to a normal CO_2 capacity was gradual and in one case was not complete in 50 minutes.

3. The changes in CO_2 capacity may be explained by the accumulation and removal of lactic acid, two processes which occur simultaneously but at different rates during and after muscular exertion. CO_2 capacity continues to fall until the rate of removal of lactic acid becomes greater than the rate of accumulation.

4. The CO_2 tension of arterial blood was increased during the first 2 minutes of exercise. As the volume of respiration increased, the CO_2 tension fell and was approximately normal after $3\frac{1}{2}$ minutes of exercise. It diminished progressively for several minutes following the exertion. The return to the original tension was gradual and was not complete 30 to 50 minutes after exercise.

5. The response to exercise is not an acapnia phenomenon. The CO_2 capacity (bicarbonate) of the blood was diminished at a time (2nd minute of exercise) when the CO_2 tension was increased and when the respiration was inadequate to remove all of the CO_2 which was produced. At no time was there an alkalosis. Even during the 2nd minute of work, there was a marked diminution in alkalinity which was progressive as the exercise continued and from 1 to 3 minutes after the cessation of exercise. Return of the reaction to normal is more rapid than the return of either CO_2 tension or capacity, but is delayed from 8 to 30 minutes.

6. The continued diminution in the alkalinity of arterial blood is attributable to the great accumulation of lactic acid with the consequent decrease in bicarbonate content. The greater respiration and the attendant lowering of CO_2 tension tend to oppose the effect of lactic acid on the reaction, but are not great enough to prevent an acidosis from occurring nor to cause a prompt return of the reaction to normal.

7. When the duration of work is $7\frac{1}{2}$ minutes, the maximum changes in CO_2 capacity, CO_2 tension, and reaction may occur during the exercise.

8. Changes in the CO_2 capacity and reaction of venous blood from an arm vein are in the same direction as those of arterial blood. The CO_2 tension, however, may remain higher than normal for 3 minutes after the end of exercise.

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Protocols.

Subject.	Date.	Time blood was taken.	CO₂ absorption curve. CO₂ tension	CO₂ absorption curve. CO₂ content.	CO₂ content of blood as drawn	O₂ content of blood as drawn	O₂ capacity.	Remarks.
	1921		*mm. Hg*	*vol. per cent*	*vol per cent*	*vol. per cent*	*vol. per cent*	
D. P. B.	Dec. 23	1 min. after exercise.	40.6	38.2	49.9	3.4		Venous blood.
				36.9	48.7	4 1		Exercise = 4,332 kgm. in 3½ min.
		3 min. after exercise.	39.9	32.0	37.7	15 3		
				32.8	36.8	14.2		
		8 min. after exercise.	39.9	31.9	35.4	13 8		
				32 0	35.5	14 0		
			59.5	38.8				
	1922							
D. P. B.	Mar. 31	Before exercise.	39.9	44 4	52 6	10 9		Venous blood.
				45 5	52 0	10 9		Exercise = 4,530 kgm. in 3½ min.
		1 min. after exercise.	40.5	37 6	47 2	5 8		
				36 7				
		3 min. after exercise.	39 9	30 5	37 6	8 5		
				30 2	37 2	8 8		
			61 1	37.6				
				38 6				
D. P. B.	Apr. 10	8 min. after exercise.	23.7	20.6	29.1	17.5		Venous blood.
				20.2	28.9	17.4		Exercise = 4,434 kgm. in 3½ min.
			55.6	33 5				
				33.3				
		19 min. after exercise.	39.3	30 1	35 9	12.8		
				30.2	35.3	12.6		
		35 min. after exercise.	40.1	38.1	43.0	11.2		
				38.1	43.8	11.5		

D. P. B.	June 21	Before exercise.	39.7	46.3	47.4	20.0		Arterial blood.
				46.2	47.2	20.4		Exercise = 7,380 kgm. in 7½ min.
		During last min. of exercise.	29.5	24 9	24.5	23.8		Blood protected against acid changes with 0.1 per cent sodium fluoride and low temperature.
				24.4	24.7	23.8		
		5 min. after exercise.	29.4	24.7	24.6	22.6		
				25.0	25.2	22.8		
D. P. B.	Oct. 24	Before exercise.	40.0	46.4	46.3	21.8	21.8	Arterial blood.
					46.1	21.5		Exercise = 3,504 kgm. in 3¼ min.
		15 min. after exercise.	38.3	34.2	32 0	21 4	22.5	Blood protected with 0.1 per cent sodium fluoride.
					31.6	21 6		Lactic acid:
								Before.................. 23.8, 25.2 mg.
								After.................. 64.4, 65.1 mg.
H. E. H.	Apr. 5	Before exercise.	39.0	49.5	54.2	19.4		Arterial blood.
				49.9	54.8	19.7		Exercise = 3,954 kgm. in 3½ min.
		1 min. after exercise.	36.0	33 6	36.6	21 3		
				33.4	36.0	20.8		
		3 min. after exercise.	36 4	31.0	31.7	21.4		
				30.9	31.8	20.7		
			57.3	38.4				
				38.6				
H. E. H.	Apr. 14	Before exercise.	29.6	44 4	51 0	19.5	21.8	Arterial blood.
				44 5	50.3	19.3		Exercise = 4,098 kgm. in 4 min.
			46.3	53 3				Second blood specimen taken during last min. of exercise.
				53 1				
		During exercise.	28.9	35.8	40.3	20.9		
				35.5	40.0	21.9		
			50.4	45.5				
				45.1				

Protocols—Concluded.

Subject.	Date.	Time blood was taken	CO_2 absorption curve: CO_2 tension	CO_2 absorption curve: CO_2 content	CO_2 content of blood as drawn	O_2 content of blood as drawn	O_2 capacity	Remarks
	1922		*mm Hg*	*vol per cent*	*vol per cent*	*vol per cent*	*vol per cent*	
H. E. H.	Apr. 21	3 min. after exercise.	39.3	33.1	31.1	21.3		Arterial blood.
				33.5	30.4	22.2		Exercise = 4,104 kgm. in $3\frac{1}{2}$ min.
		15 min. after exercise.	28.5	30.8	35.4	20.6		
				32.0	35.2	21.1		
			63.2	46.0				
				45.8				
		30 min. after exercise.	31.6	41.2	45.5	19.8		
				41.4	44.9	20.0		
			46.3	47.4				
				48.3				
H. E. H.	Aug. 10	Before exercise.	39.0	48.4	48.5	18.1	19.0	Arterial blood.
				49.0	49.2	17.8	19.1	Exercise = 3,400 kgm. in $3\frac{3}{4}$ min.
		During exercise.	37.7	41.7	40.8	19.6	20.0	Rate of pedalling slower. Weight on ergometer heavier than in previous experiments. Blood protected with 0.1 per cent sodium fluoride and low temperature.
				42.6	40.2	19.7	19.9	
		3 min. after exercise.	37.9	39.2	37.5	19.9	20.2	
				40.2	37.4	19.4		
								Lactic acid: Before .... 15.4, 13.7 mg.
								After .... 46.9, 45.5 mg.
H. E. H.	Aug. 25	Before exercise.	39.1	47.3	50.4	17.9	17.4	Arterial blood.
				47.6	51.4	17.2	17.5	Exercise = 3,545 kgm. in 3 min. 20 sec.
		During last min. of exercise.	38.6	39.3	44.0	18.8	19.4	
				40.0	43.4	18.0	19.1	

		3 min. after exercise.	38 3	38 1		18 2	18 4	Lactic acid: Before .... 15.4, 12.6 } mg. During .... 48.3, 46.9 } mg. After .... 46.9, 46.2 } mg. Blood protected with 0.1 per cent sodium fluoride.
				39 1		17 8	19 3	
H. E. H.	Oct. 27	Before exercise.	44 5	48 7	50 8	20 1	21 4	Arterial blood.
				49 2	50 2	19 9	20 8	Exercise = 3,408 kgm. in 3½ min.
		During 2nd min. of exercise.	39 2	43 9	49 7	21 6		Blood protected with 0.1 per cent sodium fluoride.
				43 8	49 5	20 8		Lactic acid: Before .... 16.8, 18.9 } mg. During ... . 46.2, 43.4 } mg.
J. E.	1921 Nov. 12	Before exercise.	39 9	44 5	44 6			Arterial blood.
				44 6	43 8			Exercise = 3,985 kgm. in 3½ min.
		50 min. after exercise.	40 3	41 3	40 2	21 1		
				40 8	40 0			
J. McL.	Nov. 30	Before exercise.	36 8	46 4	51 3	20 9		Arterial blood.
				47 6	50 6	21 2		Exercise = 3,[illegible]05 kgm. in 3½ min.
			48 9	51 7				
				52 1				
		3 min. after exercise.	22 8	27 3	32 3	22 2		
				26 9	32 7	22 6		
			34 2	30 9				
				30 9				
		8 min. after exercise.	36 6	41 1	41 6	22.1		
				41 0	41 3			

THE NUCLEOTIDES FORMED BY THE ACTION OF BOILED PANCREAS ON YEAST NUCLEIC ACID.

By WALTER JONES AND M. E. PERKINS.

(*From the Laboratory of Physiological Chemistry, the Johns Hopkins University, Baltimore.*)

(Received for publication, February 5, 1923.)

One of us has previously shown[1] that a boiled extract of pig's pancreas exerts a decomposing action on yeast nucleic acid without setting free either phosphoric acid or purine bases and it is reasonable, therefore, to suppose that the decomposition consists of the rupture of only nucleotide linkages with the formation of nucleotides.

It was also shown that in this decomposition, no titratable acidity is produced and the importance of this observation in connection with the chemical constitution of yeast nucleic acid was pointed out.

It was also reported that from the product a mixture of the mononucleotides had been prepared, but the separation of this mixture into its individual constituents was postponed. It is our purpose in the present paper to describe this separation and the identification of the individual nucleotides obtained.

Adenine Nucleotide.—Adenine nucleotide was isolated in crystalline form and did not differ in any particular from the substance originally prepared by Jones and Kennedy[2] by the oxidation of yeast nucleic acid with potassium permanganate and afterwards found by Jones and Abt[3] among the products of the hydrolysis of nucleic acid with ammonia under pressure.

Guanine Nucleotide.—This substance was isolated as the free amorphous nucleotide identical in all respects with the guanine

[1] Jones, W., *Am. J. Physiol.*, 1920, lii, 203; *J. Biol. Chem.*, 1922, l, 323.

[2] Jones, W., and Kennedy, R. P., *J. Pharmacol. and Exp. Therap.*, 1918–19, xii, 253.

[3] Jones, W., and Abt, A. F., *Am. J. Physiol.*, 1919–20, l, 574.

nucleotide originally prepared by Jones and Richards[4] by the action of yeast powder on yeast nucleic acid.

The substance was further identified by two of its crystalline brucine salts, one of which is described for the first time in the present paper.

In spite of repeated efforts which involved large quantities of material and consumed a great deal of time, we were unable to prepare the crystalline guanine nucleotide which Levene[5] describes. Levene stated that he was unable to give any specific method for its preparation.[6]

Cytosine Nucleotide.—Cytosine nucleotide was obtained by means of a crystalline lead ammonium salt which is described for the first time in this paper. This salt has confused us many times in the past, for its nitrogen percentage is almost identical with that required for free adenine nucleotide.

From this lead salt, free crystalline cytosine nucleotide was prepared, having the required composition and without doubt identical with the substance first described by Thannhauser and Dorfmüller.[7]

Uracil Nucleotide.—The isolation of this substance as a free nucleotide was not attempted because the properties of the compound apparently are not characteristic and its isolation is most difficult.[8] Following the procedure of Levene[9] we were able to prepare the characteristic brucine salt of uracil nucleotide.

As may be inferred from the descriptions in the experimental part that follows, no attempt was made to keep account of quantitative relations, but the amount of the brucine salt of uracil nucleotide which was obtained in a number of different experiments was unexpectedly small. This is a little surprising because on former occasions we have never failed to find this substance in comparative abundance. Its properties are such that it is the one of the four nucleotides least likely to be analytically lost. We will take the matter up at another time.

[4] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71.

[5] Levene, P. A., *J. Biol. Chem.*, 1919, xl, 171.

[6] Levene, P. A., *J. Biol. Chem.*, 1919, xl, 423.

[7] Thannhauser, S. J., and Dorfmuller, G., *Z. physiol. Chem.*, 1919, civ, 65.

[8] Levene, P. A., *J. Biol. Chem.*, 1920, xli, 1.

[9] Levene, P. A., *J. Biol. Chem.*, 1918, xxxiii, 229.

EXPERIMENTAL.

25 gm. portions of nucleic acid were treated with boiled aqueous extract of pig's pancreas and digested in the thermostat at 40° for 2 days. After the nucleic acid had completely disappeared, phosphoric acid was removed from the product and a crude mixture of the nucleotides was isolated by the procedure described in a former paper.

An aqueous solution of the nucleotides was made strongly alkaline with ammonia and an equal volume of absolute alcohol was added. After filtering off the precipitated ammonium salt of guanine nucleotide tightly with a pump, the fluid was treated with more alcohol in small successive portions, which produced an increasing precipitate; but after each addition of alcohol, a small portion of the fluid was filtered off and tested for combined guanine. When enough alcohol had thus been added for the precipitation of all combined guanine, the material was treated additionally with a considerable amount of alcohol and the precipitate was filtered tightly on a Buchner.

This treatment is based on the fact that the ammonium salts of the four nucleotides are precipitated from an aqueous solution by alcohol in a rather definite order, so that the material can be separated in this way into three fractions.[10]

1. The Guanine Fraction.—This is the first precipitate that was filtered off. It consists of the ammonium salt of guanine nucleotide, but does not contain any of the other three.

2. The Middle Fraction.—This is the second precipitate that was filtered off and contains the remainder of the guanine nucleotide with a small amount of cytosine nucleotide, but contains little if any adenine nucleotide and no uracil nucleotide. The fraction was rejected.

3. The Adenine Fraction.—This is the final aqueous alcoholic filtrate. It contains all the uracil nucleotide, nearly all the adenine nucleotide, and all the cytosine nucleotide except the small portion that was precipitated in the "Middle fraction." But it is free of guanine nucleotide.

[10] Jones, W., and Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 93. Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 111.

The Guanine Fraction.

The cake was several times dissolved in warm water, made alkaline with ammonia, and precipitated with an equal volume of alcohol in order to remove adherent portions of the middle fraction. The final product was dissolved in hot water, faintly acidified with acetic acid, strongly diluted with boiling hot water, and treated with enough neutral lead acetate to precipitate completely the nucleotide. The precipitate was filtered with a pump and after washing (by removal, grinding, and refiltration) until the wash water gave no ammonia with sodium carbonate in the cold, the lead salt was suspended in hot water and decomposed with hydrogen sulfide. The filtrate from lead sulfide was aerated for the removal of sulfuretted hydrogen, evaporated at 45° under diminished pressure to a thin pale yellow syrup and treated with twice its volume of alcohol. The precipitated guanine nucleotide was hardened and dried with absolute alcohol.

Brucine Salts of Guanine Nucleotide.—A solution of guanine nucleotide in hot water was neutralized with a concentrated solution of brucine in hot alcohol, and after standing over night, the thick paste of crystalline brucine salt was filtered off[11] and recrystallized five times out of hot 35 per cent alcohol.

Analysis of the brucine salts obtained by allowing the five mother liquors to evaporate at the room temperature showed that recrystallization from 35 per cent alcohol had not effected any separation. The nitrogen analyses were quite uniform, but slightly lower than the theoretical requirement.

N = 9.32, 9.35, 9.83, 9.82, 9.32 instead of 9.87

Guanine Nucleotide.—Various portions of the recrystallized brucine salt were assembled and, after removal of the brucine, the free nucleotide was recovered through the lead salt. When the aerated filtrate from lead sulfide had been concentrated at 45° to a thin syrup, the usual deposition of guanine nucleotide occurred, which was redeposited from hot water and dried with alcohol. Upon addition of alcohol to the mother liquor there occurred a copious precipitation of pure white guanine nucleotide which had the same chemical composition as the deposited substance.

[11] The filtrate is referred to below as "Original mother liquor."

I. 0.4094 gave 0.2708 $MgNH_4PO_4 \cdot 6H_2O$.
II. 0.3865 " 0.2602 "

I. P = 8.49.
II. P = 8.52.
Required P = 8.54.

Hydrolysis of the dried material by heating at 100° with 20 parts of 7 per cent sulfuric acid for various lengths of time showed; (*a*) The guanine is quantitatively set free when the heating lasts only 1 hour, but is partly converted into xanthine and lost when the heating is continued for 2½ hours. In the former case not a cloud is formed by the addition of silver nitrate to the final filtrate; but in the latter, a distinct flocculent precipitate is produced. (*b*) The phosphoric acid is liberated much more slowly than the guanine, not being completely set free in 2½ hours.

Nucleotide used.	Time of heating.	Obtained.				Final filtrate with $AgNO_3$.
		Guanine.		$MgNH_4PO_4$	P	
gm.	*hrs.*	*gm.*	*per cent*	*gm.*	*per cent*	
0.2986	1	0.1233	41.27	0.1701	6.81	No cloud.
0.3125	2.5	0.1216	38.90	0.1952	7.90	Precipitate.
Theoretical.			41.60		8.54	

Original Mother Liquor.—The small yield of brucine salt obtainable from an apparently pure nucleotide is a matter that is familiar to everyone. The following will explain this loss to a considerable extent.

The first mother liquor from which the neutral brucine salt had been precipitated was treated with two and one-half times its volume of absolute alcohol, when a voluminous snow-white crystalline precipitate was thrown out. The substance was found easily soluble in water, was acid to phenolphthalein but not to methyl orange, and had the composition required for the acid brucine salt of guanine nucleotide.

Brucine
$O{=}P - O \cdot C_5H_8O_3 \cdot C_5H_4N_5O \cdot 4H_2O$
HO

I. 0.3153 lost 0.0252 at 125°.
II. 0.3178 required 10.36 cc. of standard acid (1 cc. = 0.003646 N).
III. 0.4726 gave 0.1417 $MgNH_4PO_4 \cdot 6H_2O$.
IV. 1.0157 " 0.483 dried brucine.

	Theoretical.		Found.			
	Neutral salt.	Acid salt.	I	II	III	IV
H_2O..............	9.87	8.68	7.99			
N..................	9.87	11.82		11.83		
P..................	2.43	3.73			3.79	
Brucine	61.7	47.5				47.3

The Adenine Fraction.

The mixed nucleotides were recovered from the ammoniacal solution in the usual way through the lead salts, which were then converted into brucine salts as described under the "Guanine fraction" and the latter were recrystallized nine times out of hot 35 per cent alcohol.

Uracil Nucleotide.—The final crystalline residue had the nitrogen percentage required for the brucine salt of uracil nucleotide.

I. 0.4916 required 9.10 cc. of standard acid (1 cc. = 0.00364 N).
II. 0.2310 " 4.22 " " " " (1 cc. = 0.00364 N).

	Required for compound of		Found.	
	Cytosine.	Uracil.	I	II
N..	7.92	6.76	6.74	6.81

The alcoholic mother liquors of the nine recrystallizations from 35 per cent alcohol were allowed to evaporate at room temperature and the brucine salts recovered.

Adenine Nucleotide.—This was prepared from the brucine salts recovered by evaporating the first and second mother liquors obtained in the nine recrystallizations of the mixed brucine salts noted above and was plentifully obtained in crystalline needles which had all the characteristics of adenine nucleotide described in previous papers.

I. 0.2247 required 11.76 cc. of standard acid (1 cc. = 0.00364 N).
II. 0.2618 " 14.10 " " " "

	Required.	Found I	Found II
N	19 18	19.05	19.15

Cytosine Nucleotide.—This was prepared from the brucine salts recovered by evaporating the third, fourth, fifth, and sixth mother liquors obtained in the nine recrystallizations of the mixed brucine salts noted above. At a convenient point, the mother liquor from adenine nucleotide was joined.

The free nucleotide was prepared from the brucine salts in the usual way through the lead salt which was suspended in hot water and decomposed by the passage of sulfuretted hydrogen. But we were over careful not to pass the sulfuretted hydrogen too long and probably did not use an excess as was found later: for upon evaporation of the aerated filtrate from lead sulfide at 45° under diminished pressure, a crystalline deposit began to appear long before the solution had been sufficiently concentrated for the deposition of cytosine nucleotide. Upon recrystallization, the substance formed clusters of long transparent prisms which, from a nitrogen analysis, we supposed to be an anomolous form of the cytosine nucleotide described by Thannhauser and Dorfmüller.

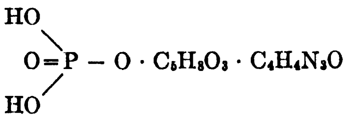
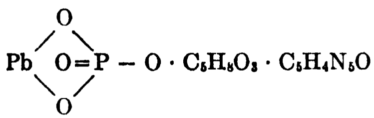
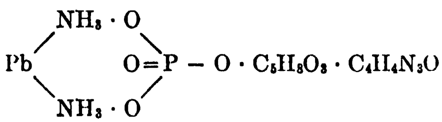
0.2023 required 7.12 cc. of standard acid (1 cc. = 0.00364 N).
N = 12.82 instead of 13.00.

But on finding that the substance contained lead we concluded that it must be a crystalline lead salt of adenine nucleotide. To make sure, the free nucleotide was prepared from the lead salt. It formed beautiful crystals like cytosine nucleotide, was anhydrous, did not give any purine material on hydrolysis, contained no easily split phosphoric acid, and had the correct nitrogen percentage.

0.3005 gave 0.2236 $MgNH_4PO_4 \cdot 6H_2O$.
0.2023 required 7.12 cc. standard acid (1 cc. = 0.00364 N).

	Required.	Found I	Found II
P	9.59	9.41	
N	13.00		12.81

The crystalline lead compound is therefore in all probability the lead ammonium salt of cytosine nucleotide. The following theoretical values will explain our temporary confusion.

	Nitrogen.	
	Theoretical.	Found.
 Cytosine nucleotide........................	13.00	12.81
 Neutral lead salt of adenine nucleotide	12.70	
 Lead ammonium salt of cytosine nucleotide...	12.48	12.82

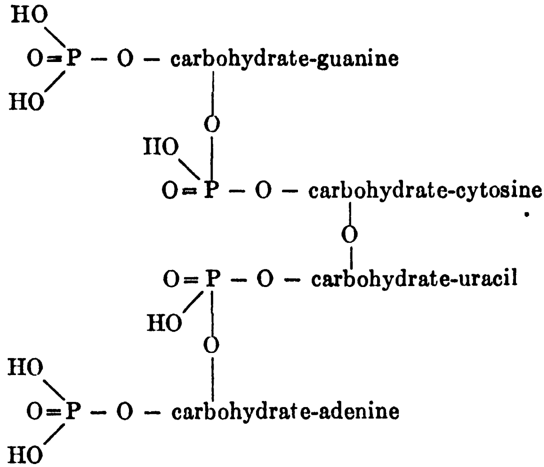
CONCLUSION.

From the experimental findings described above one is likely to conclude that the thermostable active agent of pancreas splits nucleic acid into its constituent mononucleotides. But this is not the case, for it can be shown that the end-product contains intermediate substances between nucleic acid and mononucleotides. Nevertheless, it seems certain that in this decomposition of nucleic acid by the boiled pancreas, nucleotide linkages, and no others are ruptured. When we recall that this rupture produces no titratable[12] acidity the position of at least one of the nucleotide linkages is indicated. It should be a carbohydrate linkage.

[12] The amount of increased acidity theoretically required is small, but we think we can detect one-tenth this amount.

But we have found a means of decomposing nucleic acid quantitatively into its mononucleotides and have observed that this decomposition *does* produce titratable acidity toward phenolphthalein. This circumstance indicates that one or more of the nucleotide linkages of nucleic acid bind a carbohydrate group to a phosphoric acid group.

The following formula[13] is therefore suggested.



[13] In this formula no significance is attached to the order in which the nucleotide groups are arranged nor even to their number; only to the modes of nucleotide linkage.

THE FORMATION OF NUCLEOTIDES FROM YEAST NUCLEIC ACID BY THE ACTION OF SODIUM HYDROXIDE AT ROOM TEMPERATURE.

BY WALTER JONES AND M. E. PERKINS.

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(Received for publication, February 5, 1923.)

While we are not ready to report on the matter, propriety may demand an immediate statement of the chemical method referred to in the preceding paper by which nucleic acid can be decomposed into its nucleotides in such a way that the course of the decomposition may be accurately followed.

About a year ago, Steudel and Peiser[1] announced that when a solution of the sodium salt of yeast nucleic acid is made faintly alkaline with sodium hydroxide and allowed to stand over night, guanylic acid (*i.e.* guanine nucleotide) is quantitatively liberated, leaving a complex of the nucleic acid whose examination would offer great difficulty and consume much time. This statement rather concedes the right of solving the problem to any one who thinks he knows what Steudel and Peiser's residue really is and who believes that he can acquire in a few days the experimental data to prove his opinion.

More recently, Levene[2] has repeated Steudel and Peiser's experiment. We believe that he misinterpreted the somewhat ambiguous directions of Steudel and Peiser and used much more alkali than these workers had employed; nevertheless, as will appear, he did not use enough alkali to neutralize the products formed and still maintain the alkalinity necessary for the complete decomposition of the nucleic acid. The final product therefore contained part of the initial nucleic acid together with the nucleotides formed from the other part, a condition which produced great analytical difficulty.

[1] Steudel, H., and Peiser, E., *Z. physiol. Chem.*, 1922, cxx, 292.

[2] Levene, P. A., *J. Biol. Chem.*, 1923, lv, 9.

We have been engaged with this matter for some time. If such had not been the case, Levene's results would have prevented us from entering so difficult and uncertain a field of experimentation.

We suspended 50 gm. of nucleic acid in 1,250 cc. of water and added sodium hydroxide cautiously until all the nucleic acid had dissolved, giving a solution that was neutral to litmus. 50 cc. of a 20 per cent sodium hydroxide solution were then added in excess, making the entire fluid about 1 per cent. Under these conditions the nucleic acid decomposed completely in a few hours at the room temperature and in accordance with the following statements.

1. Neither phosphoric acid nor purine bases are set free.
2. There is a progressive diminution of alkalinity (to phenolphthalein) as the decomposition proceeds. This shows the appearance of weak acid as the nucleotides are formed from nucleic acid.
3. In the beginning, the material gives a dense white precipitate both with sulfuric acid and with hydrochloric acid. After about half the time required for the complete decomposition of the nucleic acid, the product fails to give even a cloud with sulfuric acid, but forms a heavy precipitate with hydrochloric acid.
4. After a sufficient time (3 to 4 hours) neither sulfuric acid nor hydrochloric acid will form the slightest cloud. When the amount of additional alkali used is half as much as the amount stated (*i.e.* 0.5 per cent) the nucleic acid is not decomposed in any length of time.

From the product, mononucleotides can be easily and rapidly isolated and in an amount to suggest that the reaction is quantitative. We have obtained in this way unexpectedly large quantities of cytosine nucleotide without employing brucine in the procedure.

Naturally enough, the possession of a method by which nucleotides can be easily prepared opens up to us lines of experimentation which have hitherto been closed for lack of material.

A NEW OPTICAL INSTRUMENT FOR THE DETERMINATION OF HEMOGLOBIN.

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(*From the Laboratory of the Henry Phipps Institute, University of Pennsylvania, Philadelphia.*)

(Received for publication, February 17, 1923.)

In a previous article[1] the author discussed the conditions under which a satisfactory artificial color match might be obtained for hemoglobin or its derivatives. It was shown that there is in fact no satisfactory material matching oxyhemoglobin because of the depth of its absorption bands. These bands are deeper than those of any other known material of similar color. Acid hematin is a substance with a smoother absorption curve. Materials can be found with absorption curves nearly matching the curve of acid hematin and in which the corresponding physiological transmissions are even more nearly equal. Such an equivalent curve was found in one sample of a high transmission yellow signal glass. The matching color so obtained and tested has a greater uniformity in its color value, as judged by different individuals, than has any possible glass selected to match oxyhemoglobin. The hemoglobin value of 1 mm. sections of this yellow glass was carefully determined.

It is the purpose of this article to describe a hemoglobinometer which has been constructed, using this particular yellow glass of known hemoglobin value as a color standard. The principle on which the instrument is constructed is the making of a photometric match between a standard filter of 1 mm. section of this yellow glass on one side, and a sample of blood diluted with 1 per cent hydrochloric acid on the other.

All instruments for the determination of hemoglobin now on the market, with the exception of certain ones using carbon monoxide hemoglobin as a standard, and the Miescher instrument

[1] Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 465.

which matches oxyhemoglobin, either use materials as standards for which the color value is not determined with any very reassuring accuracy or the materials are not permanent in color. Of the red glass instruments, the Miescher is the only one which seems to be properly standardized. All of the red glass instruments have the inherent spectrometric errors mentioned above.

It has been our aim to produce a precision instrument for the measurement of hemoglobin. We have therefore used acid hematin as the substance to be matched because it is the hemoglobin for which the most exact photometric match can be obtained. The glass used as a standard is permanent in color and the basis on which its hemoglobin figures depend is clearly defined. In conjunction with the Technical Bureau of the Bausch and Lomb Optical Company every effort has been made to perfect the optical qualities of this instrument and, while making a colorimeter of high optical quality and precision, it has been our endeavor to provide a method of reading percentage hemoglobin directly with the least possible manipulation.

The instrument is a modified Duboscq colorimeter of compact design. The body of the instrument (Fig. 1) is of aluminum castings to which are attached the integral parts of the equipment. The rack and pinion actuating the cup table is of the precision microscope type, both cups being operated by the same pinion. To the pinion is attached a drum provided with a spiral groove in which travels a pointer fixed in the table. The calibration is along the margin of the groove on the drum. This arrangement gives a direct reading instrument calibrated in percentage hemoglobin. The individual divisions on the drum are widely spaced and permit easy reading of the result to within 1 per cent. In addition to the feature of accuracy the position of the drum is such as to permit its being easily observed by a slight shift of the eyes from the position at the eyepiece.

The prism system is of double rhomboid type, the inner reflecting surfaces of both rhomboids being silvered. The dividing line of the field is formed by the sharp edge of a silvered reflecting surface. This dividing line becomes practically invisible when the fields become the same color, the fields shade imperceptibly into one another and very delicate and accurate settings are assured.

The standard slip of colored glass is incorporated in the instrument at the top of one of the plungers. By a special process it

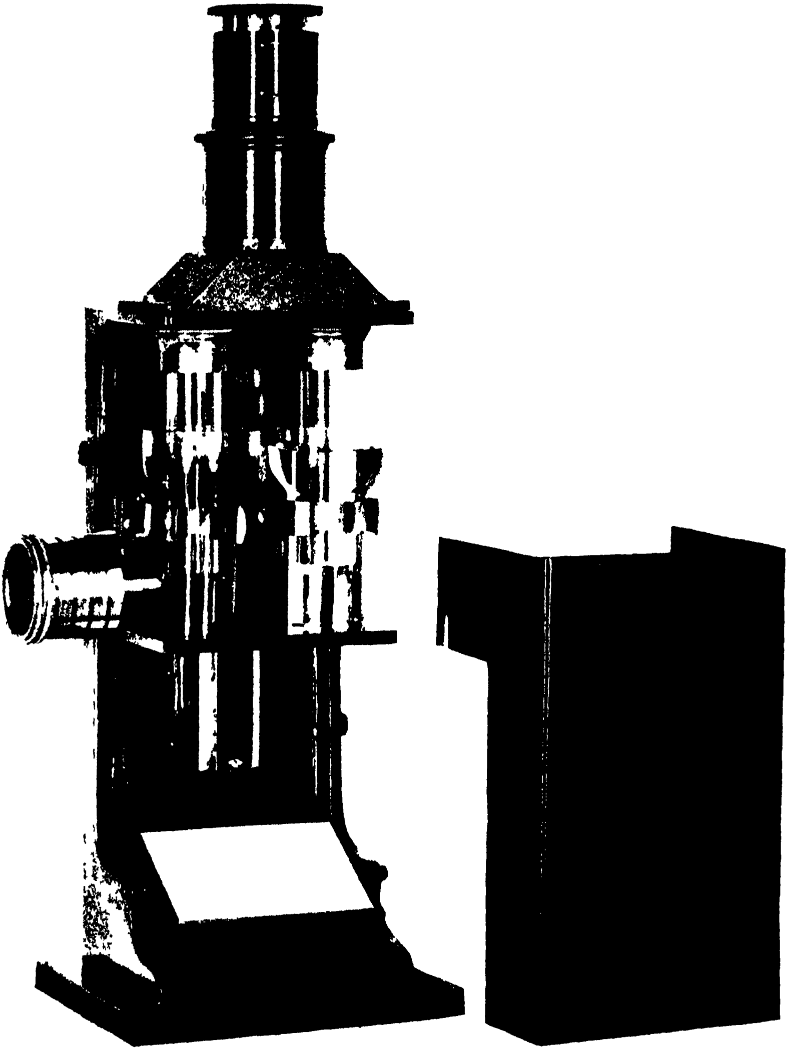

Fig. 1.

is ground and polished accurately to within $\frac{1}{2}$ of 1 per cent of the desired millimeter thickness. The cups are of clear glass

with bases of optical glass fused firmly upon them. Their size is such that they carry the volume contained in the mixing pipette and give with this amount of fluid readings over the entire scale. One of the cups is to be filled with plain water and the other with the blood preparation. The latter is made by diluting 10 c.mm. of blood to 502 volumes with 1 per cent hydrochloric acid. A suitable pipette is supplied with which to make this dilution. At this dilution readings down to 40 per cent hemoglobin may be obtained. For lower values 20 c. mm. of blood in the same pipette are used.

It is possible to adjust the drum to correct position when the plunger is on the bottom of the cup. This adjustment would be necessary if the thickness of the glass bottom of a replacement cup differed from that of the one supplied with the instrument. However, the optical glass used is so uniform that it is unlikely that a new cup will require readjustment of the drum.

The height of the instrument is 8 $\frac{15}{16}$ inches and the weight 2 lbs. 4 oz. and with the case 3 lbs. 7 oz.

The instrument is calibrated to read percentage hemoglobin. The colored glass, as originally calibrated, had a hemoglobin value equivalent to a 1 cm. column of acid hematin solution derived from a 1 in 502 dilution of blood containing 19.08 gm. of hemoglobin in 100 cc.

There were some good reasons for proposing to calibrate this instrument to read hemoglobin in grams, but customary usage determined us to give the figures in percentage and to use as a basis for the standard or normal for 100 per cent the information in the data of Williamson.[2] These data are a collection of very careful determinations of the hemoglobin content of the blood of 919 normal individuals of varying ages living in the city of Chicago. The average figure for the adult males was 16.92 gm. of hemoglobin per 100 cc. of whole blood and this is taken as the 100 per cent of our scale.

Fig. 2 gives these values of hemoglobin for varying age and sex in chart form. Smooth curves have been drawn from the data to which we refer. From the curves one can readily see the range in hemoglobin values with age and sex and the meaning which the percentage figures have.

[2] Williamson, C. S., *Arch. Int. Med.*, 1916, xviii, 505.

When whole blood is mixed with 1 per cent hydrochloric acid a yellow solution of acid hematin results. The color of this

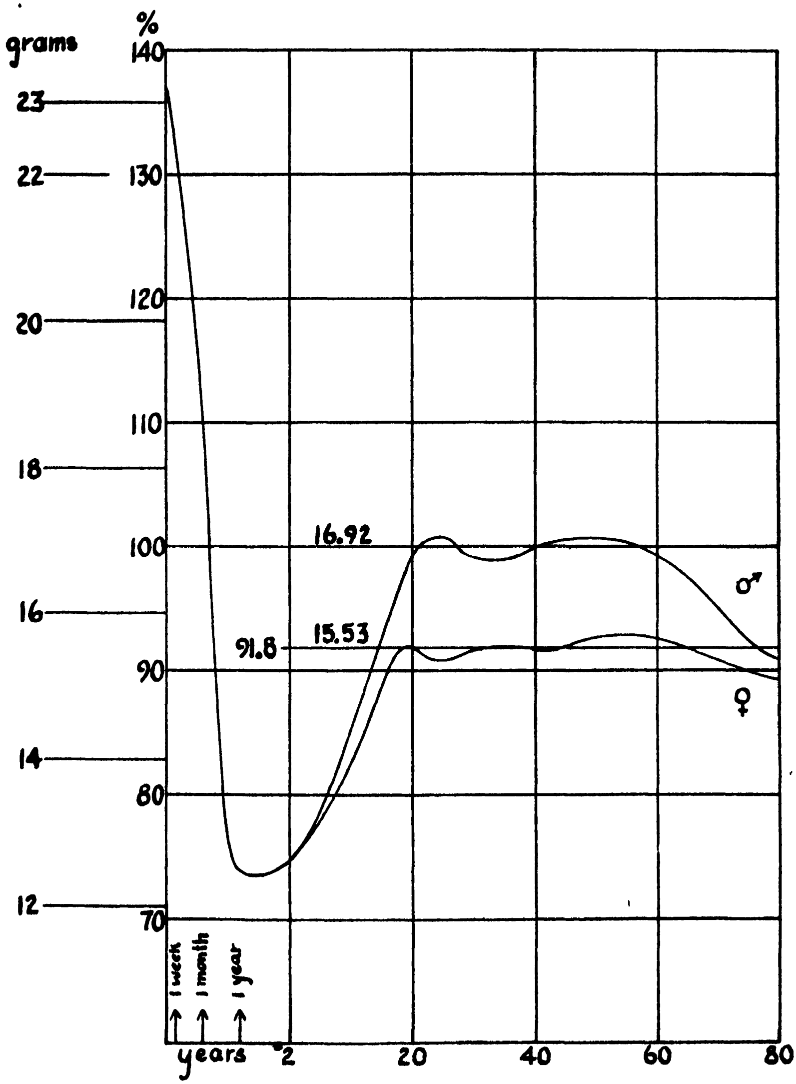

Fig. 2.

solution deepens rapidly at first and continues to deepen with time. It deepens according to a law; namely, the color depth,

at a given time after dilution, falls short of its final value by a percentage equal to $\frac{40}{t}$ where t equals the time since dilution, expressed in minutes. The instrument is calibrated to read to absolute correctness when the color is at its final depth. Theoretically, the color never reaches its final depth and from a theoretical point of view then, the reading is always a little low. However, after the solution has stood for ½ hour the readings are correct to within approximately 1 per cent. If the time elapsing between the making of the solution and the reading is short enough to make this error objectionably large, it can be corrected for by rule or by the use of a table which accompanies the instrument.

The instrument which we have produced and have described is of high optical and mechanical quality. The hemoglobin readings of this instrument are based on well known and reliable data for normals. The result is a precise instrument equipped with a scale of hemoglobin values which are well defined, therefore, permitting accurate readings in figures which in other instruments have been based on uncertain or undefined standards.

THE INFLUENCE OF RADIANT ENERGY UPON THE DEVELOPMENT OF XEROPHTHALMIA IN RATS: A REMARKABLE DEMONSTRATION OF THE BENEFICIAL INFLUENCE OF SUNLIGHT AND OUT-OF-DOOR AIR UPON THE ORGANISM.*

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The preventive influence of direct sunlight and of radiation emitted by the quartz mercury-vapor lamp upon the development of experimental rickets in rats has been demonstrated in experiments reported in previous studies (1, 2). A logical further step was to determine whether or not direct sunlight and quartz mercury-vapor lamp radiation would also prevent the development of xerophthalmia in rats fed diets which under ordinary conditions of room light would bring about the development of both rickets and xerophthalmia, and of xerophthalmia alone. If this information could be ascertained it would be a valuable contribution to the study of xerophthalmia and by analogy would suggest either the unity or the divisibility of those factors contained in cod liver oil which prevent and cure both xerophthalmia and rickets. It was desired also to determine whether or not radiations of different spectral distribution and intensity—as for example direct sunlight, quartz mercury-vapor lamp radiation, and room light—exert the same influence upon rats fed

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xerophthalmia-producing diets. It was conceivable also that complete absence of light rays might have a different effect upon experimental animals than that produced by solar radiation or of radiation considerably different from sunlight (quartz mercury-vapor lamp radiation).

Experiment 1.—On Oct. 22, 1921 fifteen albino rats, about 40 days old and weighing between 50 and 65 gm., were placed on Diet 3127. This diet, as previous experience has shown (3), will produce both rickets and xerophthalmia in growing rats. The diet has the following composition.

	gm.
Rolled oats	40
Gelatin	10
Wheat gluten	7
Dextrin	39
NaCl	1
KCl	1
$CaCO_3$	2

This diet is low in fat-soluble A and phosphorus, but contains proteins of good quality.

The animals were divided into three groups of five each. One group was to be kept in a laboratory room screened with ordinary window glass; a second group was to be kept in total darkness, excepting for the rays from a red electric light, such as is used in photographic dark rooms;[1] the third group was to be kept in ordinary room light, but was to be radiated for a certain period daily with a quartz mercury-vapor lamp.

The experiment was unsatisfactory. The animals had to be kept in a room frequented by wild rats who attacked the caged animals, sometimes with fatal results. Unfortunately, there seemed also to have been vicious animals in some of the groups. As the enfeebling effect of the diet became evident, it became more and more frequent to find animals dead and their bodies so mutilated that careful postmortem examinations, particularly of the eyes, was not possible. It was impossible at the time of the experiment to keep each animal in a separate cage.

The diet produced xerophthalmia in all the animals without regard to the varying experimental conditions. Only the animals radiated with the quartz mercury-vapor lamp failed to develop rickets as well as xerophthalmia.

[1] This bulb was lighted only when the animals were being fed or inspected.

The animals were radiated with a Hanovia quartz mercury-vapor lamp (Alpine type) and received daily exposures. For the first 36 days of the experiment the average daily exposure was 2 hours and 36 minutes. At the end of 36 days the quartz mercury arc was replaced by a new one and thereafter, for the succeeding 27 days of the experiment, the animals received an average daily exposure of 42 minutes. The exact quantitative and qualitative differences, if any, in the radiations of the old arc and the new are unknown to us. The exposure, however, to the radiation of the ultra-violet lamp was sufficient to prevent the development of rickets, but not of xerophthalmia. The general condition of the animals seemed slightly better than that of the animals in the other groups. At autopsy, however, as in the animals from the other groups, there was a striking absence of body fat. We have the impression that these animals succumbed less easily and quickly to the fat-soluble A deficiency than did the animals in the other groups. Two of the animals lived longer than did those in any other group; of course, this occurrence may have been a mere coincidence.

Those animals which were kept in ordinary room light and in complete darkness not only developed rickets and xerophthalmia, but they failed to grow appreciably and became markedly emaciated. Grossly and histologically the bones of these animals showed rickets; the disease doubtless would have been more marked had there been more growth.

Experiment 2.—On Nov. 15, 1921 another experiment was started using twenty-six albino rats, about 35 days old and weighing about 50 gm. These animals were placed on Diet 3311, on which rats develop xerophthalmia, but not rickets. This diet has the following composition.

	gm.
Rolled oats	60
Casein	15
Dextrin	21.5
NaCl	1
KCl	1
$CaCO_3$	1.5

This diet is relatively high in protein and is low in fat-soluble A.

After the animals had been on this food for 68 days the diet was changed to No. 3392 (4), which is constituted as follows:

	gm.
Rolled oats	40
Casein	5
Dextrin	52.5
NaCl	1
$CaCO_3$	1.5

This diet is also low in fat-soluble A, but poorer in protein of good quality than Diets 3311 and 3127; and the rapid development of xerophthalmia could be expected. When high protein diets are used and the proteins are of good quality the rats grow more than they do when on low protein diets and come down more slowly with xerophthalmia.

The twenty-six animals were distributed in four groups. Group 1 (six animals) was to be kept in a room screened with ordinary window glass; Group 2 (seven animals) in ordinary room light, but to receive direct sunlight whenever possible; Group 3 (six animals) in complete darkness, excepting for the rays emitted by a photographic red light, as described in the preceding experiment; and Group 4 (seven animals) in ordinary room light but to receive radiation from a quartz mercury-vapor lamp.

On the 12th day of the experiment it was realized that the animals in Group 2 could not withstand the cold to which they were exposed when placed in direct sunlight. Accordingly, this part of the experiment was given up and four of the animals from Group 2 were kept in ordinary room light and three were placed with Group 3. At the time this transfer was made these animals had received only 14 hours of direct sunlight during the period of 12 days.

In conducting this experiment the same difficulties were encountered as in Experiment 1. None of the animals developed rickets; all developed xerophthalmia (excepting a few animals who died or were killed by their cage mates in the first few weeks of the experiment). The rats showed no evidence of growth or the deposition of body fat. The animals who received ultraviolet radiation were apparently more vigorous and longer lived than the others. The daily exposure to the ultra-violet lamp radiation averaged 54 minutes daily; this exposure did not prevent xerophthalmia.

The experiments described in the foregoing paragraphs were preliminary in nature. It was desirable to investigate further the problem of the influence of varying light conditions upon the development in rats of experimental xerophthalmia. Accordingly, the following experiments were undertaken.

Experiment 3.—On Apr. 1, 1922 twelve rats, 54 days of age and varying in weight between 50 and 100 gm., were placed on the rickets- and xeroph-

thalmia-producing diet, No. 3127. Four animals were albinos and eight were mixed black and white. The rats were divided into four groups of three each. Each group was placed in a large wire cage, with separate compartments for each animal. The cages were suspended on racks and no nesting material was provided; most of the excreta dropped out of reach through the meshes of the cages. The animals could thus receive little matter extraneous to the diet either from nesting material which might have been used or from their own excreta. The first three groups of animals were subjected to the same experimental conditions of ordinary room light, darkness, and ultra-violet radiation as were detailed in the first experiment. The ultra-violet radiation was obtained from the Alpine type of Hanovia quartz mercury-vapor lamp; the animals in this group were given an exposure of 30 minutes daily at a distance of about 3 feet. The fourth group of rats was placed in direct sunlight out-of-doors for the maximum available period of sunshine each day. Some days, when the weather was inclement, the animals received no sunlight; on other days they received perhaps only 1 hour, and occasionally they received 9 hours exposure; the average was 4 hours per day. In this experiment each animal was accurately followed as an individual throughout its career and was protected from injury from its fellows. Also, the experiment was undertaken at the most favorable possible season of the year for sunlight exposure.

The striking results of this experiment are summarized in Table I and graphically shown in Fig. 1. The animals in ordinary room light developed xerophthalmia earlier than those of any other group. They all weighed less at death than when placed on the diet. At autopsy they showed macroscopic rickets; *i.e.*, deformity of thorax, enlargement of the costochondral junctions, fractures of the ribs, and widening and irregularity of the femoral metaphyses. The eyelids were closed with purulent exudate; there was marked periorbital swelling, thickening of the lids, and corneal inflammation. The coats of the animals were rough; the rats were markedly stunted and undernourished and there was no deposit of subcutaneous or mesenteric fat. Observation of the rats kept in darkness revealed conditions identical with those just described. The development of xerophthalmia in them occurred somewhat later. The animals receiving ultra-violet radiation remained free from xerophthalmia longer and lived longer than those in ordinary room light and in darkness, but the xerophthalmia itself, however, was in no way different. These animals did not develop rickets and while their bodily states, as manifested by their weights and behavior, may be taken as slightly better than

TABLE I.

Diet 3127.

Days of exposure.	Ordinary room light. 3 rats.			Darkness. 3 rats.			Ultra-violet radiation. 3 rats.			Sunlight. 3 rats.		
	Albino.	Mixed.	Albino.	Albino.	Mixed.	Mixed.	Mixed.	Mixed.	Mixed.	Mixed.	Albino.	Mixed.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	61	53	52	97	67	100	94	82	51	83	101	82
30	*	*	*									
35		44 D.										
38				†	*	*						
42									*			
47	67.5		47.5	100	73	121	109	117	53	94	117	117
54			40.5 D.									
56	54 D.											
61									47 D.			
70				71 D.	51 D.							
74						90 D.						
76							*	*				
79								121 D.				
100							85 D.			94 D.		
160											‡	†
181											§	
187												115 D.
192											¶	
200											*	
207											117	

D. indicates that the rat died.
* Xerophthalmia.
† Recurring mild xerophthalmia until death.
‡ Mild xerophthalmia.
§ Eyes are all right.
¶ Sunlight discontinued.

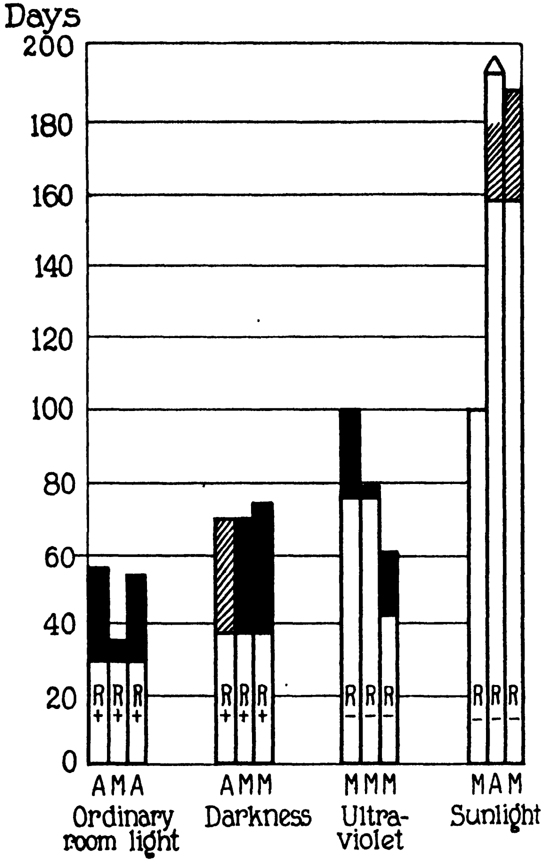

Fig. 1. Chart showing certain effects of ordinary room light, darkness, ultra-violet radiations, and direct sunlight upon rats fed a diet (No. 3127) which under ordinary laboratory conditions produces rickets and xerophthalmia.

The height of the columns indicates the days on the diet of each rat at death; the clear portion of the columns indicates the period of freedom from xerophthalmia; the black portion of the columns indicates the period of progressive xerophthalmia; the cross-hatched portion of the columns indicates the period of mild recurring xerophthalmia; the pointed tip on the columns indicates that the animal was living at the time represented by the height of column. A represents albino rat; M, mixed rat; B, brown rat; R+, rickets demonstrated at autopsy; R–, no rickets demonstrated at autopsy. The animals were 54 days old when placed on the diet, Apr. 1, 1922. When given, ultra-violet radiations from an Alpine lamp of 30 minutes exposure were given daily. Exposure to direct sunlight averaged 4 hours daily.

those of the animals in the two groups already described, it is certain that radiation from the quartz mercury-vapor lamp in a dosage more than sufficient to prevent the development of rickets did not prevent, or markedly delay, the development of xerophthalmia.

When the data from the sunlight group of animals are studied, however, very striking facts are noted. The first rat to die weighed more at death than when the experiment was begun. This animal's eyes apparently never showed xerophthalmia; the cause of death was undetermined. The second animal in this group was still living, November 1, 1922, 214 days after the experiment was begun. For the short period of time from the 160th to the 180th day this animal had recurring attacks of mild xerophthalmia, but until the 160th day the eyes were apparently normal. The xerophthalmia was manifest by slight exudation and periorbital swelling; these signs ultimately disappeared. This rat developed xerophthalmia 10 days after sunlight exposure was terminated and the xerophthalmia became progressively more severe. The third rat in this group likewise did not develop xerophthalmia until the 160th day on the diet and then only in a very mild form, as just described for the second animal. This animal died on the 187th day of the experiment. The postmortem examination revealed a pyonephrosis secondary to a ureteral calculus; this infection was apparently the cause of death; only very mild xerophthalmia was present.

Apparently, xerophthalmia in the rats in this group was just submerged, so to speak, ready to appear upon the withdrawal of the protecting influence of sunlight. When weighed on the 47th experimental day each rat had gained in weight; the weights of the three animals taken later, 53rd, 167th, and 140th day, respectively, showed no change. This fact, as well as the appearance and general behavior of the rats, indicated that they had approached a limit in their development, and beyond the point attained the influence of sunlight could not force them. It is apparently equally true, however, that so long as the sunlight was allowed to exert its influence, there was no receding from the goal attained, provided no intercurrent disease occurred.

Experiment 4.—It was also desirable to repeat the experiment of subjecting rats on a single deficiency diet, *i.e.* a diet producing only xeroph-

thalmia under ordinary laboratory conditions, to the effects of ordinary room light, darkness, ultra-violet lamp radiation, and direct sunlight. On Apr. 20, 1922 eighteen rats (albinos, mixed black and white, and brown animals) 50 days of age and weighing between 57 and 79 gm. were placed on Diet 3311 and subsequently, within 10 days, on Diet 3392. One group of five animals was kept at ordinary room light; a second group, composed of four animals, was kept in darkness; a third group, composed of four animals, was exposed to 30 minutes daily radiation with the quartz mercury-vapor lamp; and a fourth group, of five animals, was given a maximum daily exposure to direct sunlight out-of-doors, averaging 4 hours per day throughout the experimental period. Each group of animals was kept in a suspended, all metal cage composed of three compartments; some of the compartments contained two rats and some only one.

The results of this experiment are summarized in Table II and in Fig. 2. The animals kept in ordinary room light, in darkness, and exposed to ultra-violet radiation showed an initial gain in weight; this gain was neither continued nor even maintained, and at autopsy there was found no deposition of subcutaneous or abdominal fat. Indeed, the animals were markedly malnourished and stunted. All of these animals developed xerophthalmia with remarkable constancy at about the same time (40th day) and all lived about the same number of days on the diet (60 to 75 days). The xerophthalmia of some of these animals was of an extremely severe grade with ulceration of the eyeball. At autopsy there was no macroscopic evidence of rickets.

In striking contrast were the results obtained with the animals exposed to direct sunlight. Only with the second animal in the group were the results comparable to those just described. This rat developed xerophthalmia early and the disease became extremely severe. The animal was killed on the 91st day of the experiment. The third and fourth animals in the group developed xerophthalmia relatively late (about the 90th day) and only a few days before their death. These rats with the second animal, although they were the most severely affected in the group, lived approximately 20 days longer than the animals in the ordinary room light, darkness, and ultra-violet groups. The first animal in the sunlight group was living November 1, 1922, 194 days after being placed on the diet. This animal showed no signs of xerophthalmia while being exposed to sunlight. Only after exposure to sunlight was terminated (in 10 days), did xerophthal-

TABLE II.

Diets 3311 and 3392.

Day of exposure.	Ordinary room light. 5 rats.					Darkness. 4 rats.				Ultra-violet radiation. 4 rats.				Sunlight. 5 rats.				
	Albino.	Mixed.	Albino.	Mixed.	Albino.	Albino.	Mixed.	Mixed.	Albino.	Mixed.	Albino.	Mixed.	Albino.	Brown.	Albino.	Brown.	Albino.	Albino.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	66	69	69	57	88	78	72	66.5	69	65	63	72	70	70	74	69	79	71
25												*						
35	125	125	138	112	178	114	135	123.5	126	129*	110	115.5	127.5	117	125*	105	131	115
41	*		*	*			*	*										
42													*					
47							89 D.				*							
51		*			†	*			*									
55									88 D.									
57			97 D.	78 D.														
61								104 D.										
64		101 D.																
68						90.5 D.				98 D.			88 D.					
69											82 D.	84 D.						
76					D.													

77	81																	
	D.																	
89															87‡			
91																	*	
96																*		
98																	96	
																	D.	
102																69		
																D.		
165																		101
																		D.
171														§				
180														*				
187														118				

D. indicates that the rat died.
* Xerophthalmia.
† Questionable xerophthalmia.
‡ Killed.
§ Eyes are all right; sunlight discontinued.

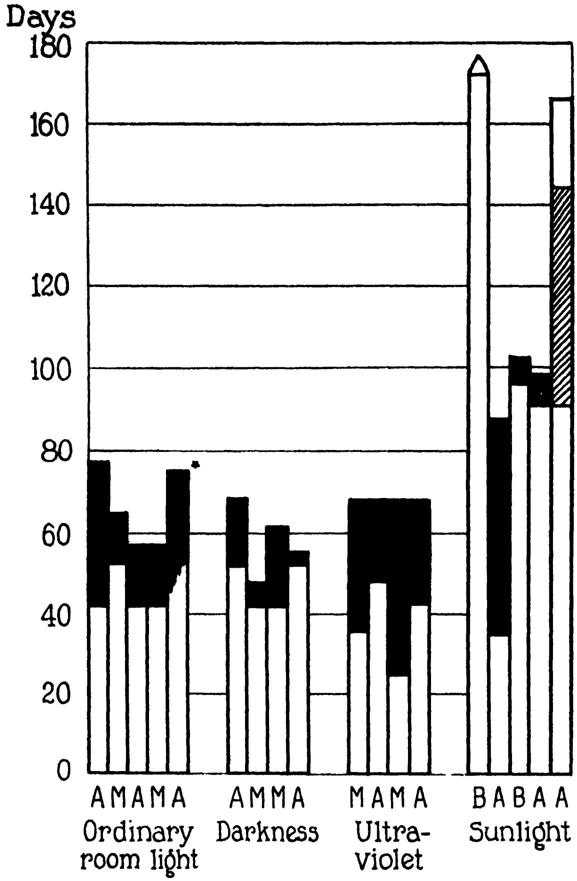

FIG. 2. Chart showing certain effects of ordinary room light, darkness, ultra-violet radiations, and direct sunlight upon rats fed diets (Nos. 3311 and 3392) which under ordinary laboratory conditions produce xerophthalmia.

The height of the columns indicates the days on the diet of each rat at death; the clear portion of the columns indicates the period of freedom from xerophthalmia; the black portion of the columns indicates the period of progressive xerophthalmia; the cross-hatched portion of the columns indicates the period of mild recurring xerophthalmia; the pointed tip on the columns indicates that the animal was living at the time represented by the height of column. A represents albino rat; M, mixed rat; B, brown rat; *, it is not known on exactly what day this rat developed xerophthalmia. The animals were 50 days old when placed on the diet, Apr. 20, 1922. When given, ultra-violet radiations from an Alpine lamp of 30 minutes exposure were given daily. Exposure to direct sunlight averaged 4 hours daily.

mia develop. The rat was vigorous and gained in weight. The fifth rat had recurring attacks of slight xerophthalmia (exudation and periorbital swelling) from the 90th to the 144th experimental day. From that time until death, on the 165th experimental day, the eyes seemed to be normal. No cause of death was determined.

In this group is seen again the powerful influence of sunlight in protecting the animals from xerophthalmia, even on a diet more defective than that used in Experiments 1 and 3. The xerophthalmia-producing potency of the diet was greater and the need for protection more necessary than with Diet 3127. This protection was not so effectively afforded as the early and extreme xerophthalmia in the second animal shows. These rats made an initial gain in weight, but only in one instance was this gain maintained; all of the animals, with one exception, however, weighed more at death than when the experiment was begun. They did not reach the plateau of constant weight attained by the sunlight group of rats on Diet 3127. Nevertheless, the long delay in the development of the disease in the third and fourth rats, its submerged nature in the fifth animal, and its actual prevention in the first, testify to the protective influence which sunlight exerted upon these animals.

DISCUSSION.

Consideration of these experiments gives rise to various ideas and permits of certain conclusions.

The rats confined in darkness developed xerophthalmia after approximately the same incubation period, so to speak, as that of the animals kept in ordinary room light. Their growth and well being seemed about the same and the period of life was approximately that of the animals on a corresponding diet but subjected to ordinary room light.

The animals kept in ordinary room light, but treated with ultra-violet lamp radiation, did not differ materially from the animals on the corresponding diet in ordinary room light and in darkness. Radiation with the quartz mercury-vapor lamp in daily dosage of 30 to 60 minutes—a dosage more than sufficient to prevent the development of rickets—does not prevent or markedly delay the development of xerophthalmia. The general

bodily condition of some of these rats may have been slightly better than that of the ordinary "room light" and "darkness" animals. The ultra-violet lamp experiments are open to the criticism that the exposures may have been either too long or too short, but it is apparently true that the radiation from a quartz mercury-vapor lamp, although it supplies in some manner the equivalent of a dietary factor which promotes the formation of normal bone, does not furnish, to any great degree at least, the equivalent of a dietary antixerophthalmic factor.

The animals kept in direct sunlight, however, exhibited marked differences from those in the other groups. The data from the sunlight experiments furnish conclusive evidence that the experimental conditions to which the animals were subjected promoted, in some manner, bodily vigor and longevity, and delayed (with one exception) and apparently occasionally prevented entirely the development of xerophthalmia. The growth of the "sunlight" animals reached a certain point, and although sunlight was not able to force the animals farther, the development attained exceeded that of any animal in the other groups and in many instances was maintained. The rats in "sunlight" (with one exception) did not develop xerophthalmia until the animals in ordinary room light, darkness, and radiated with the ultraviolet lamp had died; moreover, in the "sunlight" animals in which xerophthalmia did appear, it was not in an aggravated form and showed a tendency towards spontaneous recovery. In observing some of these rats the impression was that they were just on the verge, so to speak, of developing xerophthalmia; the occurrence in three animals of recurring mild xerophthalmia was very suggestive of this hypothesis. In the case of two of the rats exposed to direct sunlight no signs of xerophthalmia became manifest after so long a period on the defective diet that attempts to produce the condition were terminated by ending the exeriments.[2] If sunlight could have been controlled so that it

[2] It is possible that the younger the rats the more effective the protective influence of sunlight seemed to be. Drummond and Coward (5) state that: "Evidence has been produced which tends to show that the requirements of the growing rat for vitamin A become less as the animal approaches maturity . . . . and our experience leads us to believe that the amount of vitamin which must be supplied to a rat in order to restore growth which has been inhibited by feeding on the deficient basal diet, will be inversely proportional to the weight of the animal."

was constant day after day, it seems possible that the animals might have been permanently protected from xerophthalmia. The influence of the duration of exposure and of the very great intensity of radiation are factors in sunlight experimentation difficult of accurate evaluation but undoubtedly of the greatest importance. The experiments with sunlight were not perfect; in fact, in this climate no perfect experiments with sunlight are possible.

Moreover, our experiments with sunlight were complicated by the presence of other factors, whose influence is as yet unknown. No experiments involving sunlight have demonstrated that the effective agent has been the sunlight alone. It is possible that the factors which are associated with exposure to direct sunlight out-of-doors have been responsible in whole or in part for the results usually attributed to the influence of the sun's rays. In our experiments there are no available data as to the exact nature of the operative factors. The temperature to which the animals were subjected in direct sunshine in the summer months was certainly higher at times than that to which the experimental animals in the other groups were exposed. In the spring and autumn months the temperature at certain hours of the day was lower than that of the laboratory.[3] Furthermore, the sunlight

[3] Hess, Unger, and Pappenheimer (2) treated groups of rats on a rickets-producing diet with the carbon arc lamp at room temperature, and at 29, 23, and 18°C. The exposures were for 3 minutes at a distance of 3 feet—an exposure sufficient at "room temperature" to prevent rickets. Exposures to temperatures lower than "room temperature" were accomplished by placing the animals over ice. It is important to note that the animals were subjected to the various temperatures only while they were being radiated. The observers report that "fully as great protection was afforded at 18°C. as at 29°C., almost no rickets developing in groups of rats exposed at either temperature." It is inconceivable that the reactivity to light of an animal living at room "temperature" could be affected by a fleeting (3 minutes) change (maximum of 10°C.) in the temperature, not of the animal, but of its environment or that such experiments could yield any true test of the influence of the temperature of the environment on the organism. It is quite possible that temperature has little influence on the process by which lime salts are deposited in bone, but such experiments as the one just described seem well nigh valueless as a means of deciding this important question. The statement of Hess (6) in regard to the protective action of light waves in the prevention of rickets, "Nor can the physiological effect be attributed to temperature," is not justified from the experiment.

animals were in out-of-door air which was subject to possible change—chemical, physical, or both—by solar radiation and perhaps other unsuspected agencies which could not be operative in the laboratory. Whether these or unknown factors (acting singly or in combination with each other or with sunlight) or sunlight alone brought about the striking results here reported is undetermined. However, these imperfections in our knowledge do not detract from the remarkable fact brought out in bold relief by these experiments, namely *that sunlight and out-of-door air with such factors as may be associated with them have wonderful health and life-giving powers which enable the animal to adapt itself with a considerable degree of success to adverse environmental conditions.*

It may be well to point out that although both quartz mercury-vapor lamp radiation and direct sunlight prevent and cure rickets, their influence upon the development of xerophthalmia is quite dissimilar. There are marked differences in the two radiations and the factors associated with exposure to them. The more favorable action of direct sunlight may be attributable to these differences, either one or several. Attention may be called to the absence in direct sunlight of the far ultra-violet, to the presence of wave-lengths not present in quartz mercury-vapor lamp radiation, to the long duration of the exposure, to the great intensity of the radiation, to the out-of-door air, and to other factors attendant upon sunlight experimentation.

Though certain of our experiments indicate that sunlight and fresh air supplied in sufficient quantity over a sufficient length of time might completely protect rats from xerophthalmia, there are reasons for thinking that the protective action of fresh air and sunlight has limitations. The rats on the single deficiency diet were not so well protected against the development of xerophthalmia as the rats on the doubly deficient diet. The diet with the single deficiency was more powerfully xerophthalmic than the diet with the double deficiency. In neither set of animals was sunlight able to protect completely all the animals. Then in the case of some of the rats, sunlight and out-of-door air were able to keep the disease just at the surface or only slightly submerged. In one or two of the animals the xerophthalmia seemed to appear and disappear and in one or two others to

persist without becoming definitely better or worse. Then, finally, the two rats which failed to show xerophthalmia in the sunshine and fresh air after 4 to 5 months subjection to the faulty diet, developed the disease in so short a period of time after the experiment was terminated as to make it necessary to think that in those animals sunlight and fresh air just succeeded in keeping the xerophthalmia from becoming manifest.

It is interesting in this connection to inquire into the nature of xerophthalmia and into possible explanations of the partially protective influence of sunlight. Investigations of Wason (7), Yudkin and Lambert (8), and Mori (9) indicate that xerophthalmia is not a specific affection limited to the eye; apparently, when the condition occurs the metabolism of the whole body has been disturbed. The lacrimal, palpebral, and salivary glands show pathological changes and signs of diminished activity; in some cases the testes show fibrosis and evidences of diminution of function. It may be that all or most of the glands of the body have their function impaired and indeed that the general bodily metabolism is adversely affected. Our own observations indicate that growth of the skeleton ceases. We should suppose that the eye becomes affected and shows early evidences of the disease because of the extremely delicate structure of this organ. The cornea is the only highly specialized tissue on the surface of the body; when the lacrimal gland ceases to perform its function and changes take place in the mucous membrane of the lids, the cornea can no longer maintain its nutrition and becomes diseased. We cannot be certain that the development of xerophthalmia occurs in just this manner, but we can be certain, however, that when an animal is deprived of fat-soluble A the resulting condition is one in which the metabolic processes of the animal are adversely affected and xerophthalmia is merely the striking reaction on the part of the eye to a condition in which the whole body has shared. In view of these facts it does not seem so remarkable that sunlight and out-of-door air exert an inhibitory influence upon the development of xerophthalmia. Sunlight and out-of-door air supply something which raises the level of cellular function; they are not sufficient, however, to alter the activities of the body so as to compensate completely for those defects in the diet which bring about the development of xerophthalmia and its attendant bodily state.

The effects of cod liver oil and of light and out-of-door air on xerophthalmia and rickets afford interesting contrasts. The influence of cod liver oil and of sunlight on the growth, calcification, and nutrition of bone seems to be almost, if not quite, identical. Our experiments on xerophthalmia, however, indicate a difference in the effects of cod liver oil and of sunlight and out-of-door air. Cod liver oil apparently protects the rat against the development of xerophthalmia more easily than against the development of rickets; radiant energy, on the other hand, protects the animal more easily from rickets than from xerophthalmia; cod liver oil completely prevents the development of both rickets and xerophthalmia; light prevents completely the development of rickets, but does not always prevent the development of xerophthalmia. Radiant energy seems to contain the equivalent of the unknown factor in cod liver oil which prevents the development of rickets, but not to contain at all or only to a very slight degree the equivalent of the other factor in cod liver oil which prevents the development of xerophthalmia. Possibly the action of light in delaying or preventing the development of xerophthalmia is analogous to the rôle of protein in xerophthalmia-producing diets as observed by McCollum and his coworkers.[4] The protein in the diet has no specific influence for or against the development of xerophthalmia; if, however, the diet is defective in fat-soluble A, xerophthalmia can be made to develop more rapidly if the protein is insufficiently supplied or of poor quality than if abundantly supplied and of good quality. The protein of good quality, though exerting no specific influence, aids metabolic processes so that the animal is able to offer more resistance to its unfavorable environment and thus postpone the development of the disease. In fact, animals in a condition of nutritional instability may be protected against a specific type of breakdown by enhancing the quality of the diet with respect to any one of several factors. Similarly, sunlight may lack a specific property necessary for the prevention of xerophthalmia, but exert a favorable influence by improving cellular function with the result that the animals yield slowly, if at all, to the development of the malady.

[4] Personal communication.

At the outset of these experiments it occurred to us that the development of xerophthalmia might be influenced in some way by a disturbance in the relationship between radiations of different wave-lengths; that is, in the absence of light of certain wave-lengths, light of other wave-lengths might actually cause the disease. A parallelism with rickets will make this conception clear. Rickets is apparently a disturbance of the salt equilibrium of the body (involving particularly the calcium and phosphorus salts) associated with the *absence* of certain radiations (or factors closely linked with ultra-violet radiation and direct sunlight) or an unidentified organic factor in the diet. In rickets there is at present no evidence that in the absence of these radiations and the unidentified organic factor there are radiations which actually *promote* this disturbance of the salt equilibrium. In other words, there is as yet no evidence in so far as rickets is concerned that between light radiations of different wave-lengths there exists an antagonistic action. The antagonistic action, however, of radiations of different wave-lengths upon cellular activity has apparently been established; for example, rays shorter than $330m\mu$ promote lymphocytosis, whereas rays from 330 to $390m\mu$ bring about lymphopenia (10). The experiments here reported, however, do not demonstrate the association of xerophthalmia with a disturbance of light balance. If the animals were exposed to light from which most of the ultra-violet rays were filtered (ordinary room light), xerophthalmia developed; if the light was rich in rays of both the near and far ultra-violet (quartz mercury-vapor lamp radiation), xerophthalmia developed, if the light exhibited the entire solar spectrum (direct sunlight), xerophthalmia was not completely checked; and if no light at all was present, xerophthalmia developed. It cannot be inferred that darkness is equivalent to a possible balance of favorable and unfavorable radiations; that is to say, darkness is not comparable to a medium in ionic equilibrium, but rather to a medium in which no ions are present.

Though our experiments are only of value from the standpoint of analogy, the implication from them supports the view that there are in cod liver oil at least two distinct factors, one antirachitic and the other antixerophthalmic. Sunlight and fresh air can compensate for the absence of the one, but not completely for the absence of the other.

SUMMARY.

1. Young growing rats were placed on a rickets-xerophthalmia-producing diet (No. 3127). The animals were divided into four groups, each of which was subjected to a different condition of of light; namely, ordinary room light (window glass screened sunlight), darkness, ordinary room light plus ultra-violet lamp radiation (30 minutes exposure per day), and direct sunlight (average exposure 4 hours per day).

2. Similarly, other rats were placed on Diet 3392, producing xerophthalmia alone. These animals also were divided into four groups and treated in the same manner as those on the diet producing both rickets and xerophthalmia.

3. Diet 3127 was low in phosphorus and in fat-soluble A; its proteins were of good quality. Diet 3392 was low in fat-soluble A and in proteins of good quality; this diet was more powerfully xerophthalmia-producing than Diet 3127.

4. On both diets the rats in the ordinary room light, darkness, and ultra-violet lamp groups developed xerophthalmia early, ceased to grow, became markedly emaciated, and died.

5. On the doubly deficient diet (No. 3127) the animals subjected to ultra-violet lamp radiation and sunlight did not develop rickets; the rats in the ordinary room light and darkness groups developed rickets.

6. Radiation with the quartz mercury-vapor lamp for 30 to 60 minutes daily did not prevent the development of xerophthalmia, but in some animals promoted bodily vigor to a very limited degree.

7. The rats exposed to direct sunlight (with one exception) never developed xerophthalmia until the animals in the other groups had developed the disease and died. In some of these "sunlight" rats xerophthalmia developed late and the animals died; in others the disease was manifested late also but in recurring mild attacks; in still others it occurred in a mild form from which there was spontaneous recovery. Two of the rats never developed xerophthalmia at all; one of these animals and one in which there was spontaneous recovery was still living and free from xerophthalmia when the experiment was terminated, 5 months after it was begun. None of the animals had rickets.

8. Xerophthalmia, which occurred in the animals exposed to direct sunlight, was more severe in the rats on Diet 3392 than in those on Diet 3127.

9. The rats exposed to sunlight made an initial gain in weight which in the animals on Diet 3127 was maintained and which the rats on Diet 3392 never entirely lost.

10. Exposure to direct sunlight protects rats from xerophthalmia to a limited degree, dependent in part at least on the extent of the dietary deficiency in fat-soluble A and proteins of good quality (and perhaps other factors) and upon the duration and constancy of exposure. In some animals the disease develops regardless of sunlight, in others it is on the surface or just submerged, while in a few it never develops at all.

11. In this paper when the expression "exposure to direct sunlight" is used it is inclusive of all that is ordinarily associated with sunlight exposures. Radiant energy itself, changes in the atmosphere by solar radiation, variations in temperature, out-of-door air—these and factors unknown to us acting singly or in combination may be responsible for the results usually attributed simply to "sunlight."

12. Sunlight possibly exerts no specific antixerophthalmic influence, but acts by raising the level of the cellular activity of the organism to a point where the onslaughts of the disease are held in check or allowed to advance very slowly and with relatively little disturbance. It may be true that the nearer the animal to maturity the less vulnerable it is to the effects of a xerophthalmia-producing diet.

13. These experiments bring out above all else that sunlight, with such factors as may be closely linked with it, has wonderful power to promote health, bodily vigor, and longevity in animals otherwise unable to adapt themselves to markedly adverse environmental conditions.

14. Previous experiments have shown that the unknown factor in cod liver oil which promotes the normal formation of bone (and in that sense may be spoken of as antirachitic) has an equivalent in sunlight. The experiments herein reported indicate that sunlight does not contain at all or only to a very slight degree the equivalent of the antixerophthalmic factor in cod liver oil.

15. By analogy these experiments suggest that there are in cod liver oil at least two distinct factors: (1) preventive and curative of rickets, and (2) preventive and curative of xerophthalmia. Sunlight can compensate for the absence of the one, but not completely for the absence of the other.

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INVESTIGATIONS ON THE IMMEDIATE EFFECT OF HEAVY EXERCISE (STAIR-RUNNING) ON SOME PHASES OF CIRCULATION AND RESPIRATION IN NORMAL INDIVIDUALS.

III. EFFECT OF VARYING THE AMOUNT AND KIND OF EXERCISE.

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INTRODUCTION.

In two previous publications (1, 2) experiments were reported showing that exercise with the legs (stair-running, one flight five times) usually produced a marked fall in the oxygen content of blood drawn from a cubital vein within about 1 minute after the completion of the exercise. Samples of blood drawn a few minutes later showed either high or normal values for the oxygen content. The carbon dioxide content of the blood was found to decrease from 5 to 10 volumes per cent in the first few minutes after the completion of the exercise. It was pointed out that a certain similarity existed between the results in our experiments and some of the results obtained by Lindhard (3) in his investigations on the effect of static exercise.

In this paper we shall report some experiments in which we varied the amount and kind of exercise. The technique is the same as that previously described (1).

EXPERIMENTAL RESULTS.

All the experiments were performed on the same subject (E.M., one of the authors). In several previous experiments (see No. 2, Table I, Paper I), this individual always showed the same reaction to heavy exercise (running up and down one flight of stairs five times); namely, a marked fall in oxygen content of the venous arm blood within 1 minute after the completion of the exercise.

TABLE I.

Oxygen Content and Oxygen Unsaturation of Venous Blood Drawn from the Arm during Rest and after Various Kinds and Amounts of Exercise.

All the experiments were performed on the same subject (E. M.)

Experiment No.	No. in protocol.	Total oxygen-combining power of blood.	Oxygen content of venous blood drawn from arm.	Oxygen unsaturation of venous blood drawn from arm.	Pulse rate.	Respiration.	Conditions.
		vol. per cent	*vol. per cent*	*vol. per cent*			
1	B 21	19.41	13.42	5.99	72	11	Rest.
		19.41	5.90	13.51	136	32	Exercise. Stair-running five times.
2	B 23	19.69					Rest.
		19.69	5.51	14.18			Exercise. Stair-running five times.
3	B 18	17.74	11.38	6.36			Rest.
		17.74	4.60	13.14	112	28	Exercise. Stair-running three times.
4	B 17	19.41	12.12	7.29			Rest.
		19.41	6.62	12.79	108	28	Exercise. Stair-running once.
5	A 9				80	15	Rest.
		19.68	17.57	2.11	80	18	Exercise. Walked slowly up and down one flight of stairs twice.
6	B 19	18.41	14.09	4.32	78	11	Rest. Sitting quietly on a bicycle for 10 min., the left arm resting on a table at the side of the body.
			14.68	3.73	156	36	Exercise. Sample drawn after 2½ min. exercise on a bicycle, with brakes. The arms were quiet.
7	B 20	19.69	16.46	.3.23	136	34	Rest.
							Exercise. Rode a bicycle, with brakes, for 2 min. Arms were kept quiet.
8	A 5	20.55	17.18	3.37			Rest.
		20.70	17.33	3.37	132		Exercise. Sitting posture, using right arm, lifted 16 kilos 25 cm. from floor twenty-eight times. Less than ½ min. after exercise, two samples were drawn simultaneously from left (3.37 volumes per cent) and right arm (4.98 volumes per cent).
		20.70	15.72	4.98			

In the first four of the present experiments (Table I), the number of times the subject ran up and down stairs was varied from five in the first two experiments, to three in the third experiment, and to one in the fourth. Apart from these variations the conditions were the same. In all four experiments a low oxygen content was found in the cubital blood after the cessation of exercise. Running up and down a flight of stairs once produced the same effect on the oxygen content of the venous blood as did running up and down five times. The effect on the pulse and respiration rates was markedly less after running once than after running five times. Also, the subject was not as tired after the exercise in Experiment 4, as after running up and down three or five times; furthermore, it took a shorter time for him to recover. In Experiment 5, the subject walked slowly up and down a flight of stairs twice in 89 seconds. The cubital blood drawn immediately afterward showed a high oxygen content (17.57 volumes per cent). In Experiment 6 also, the exercise was performed with the legs, but instead of running up and down stairs the subject rode a bicycle. After he had been sitting quietly on the bicycle for 10 minutes with his left arm resting in a horizontal position at the side of the body, a sample of blood was drawn. After that he rode the bicycle (which had a brake on) as fast as he could for 2½ minutes, after which time the pulse and respiration rates increased to 156 and 36, respectively—an increase above that usually found after running up and down a flight of stairs five times. In a sample of blood, drawn immediately after the exercise, no decrease in oxygen content was found. The same result was obtained under similar conditions in Experiment 7. In Experiment 8, the subject was sitting on a chair. A sample of blood was drawn from the right arm (hanging down) after 10 minutes rest. Then, with his right arm, he lifted 16 kilos 25 cm. from the floor, twenty-eight times. Within half a minute after the exercise was finished, two samples of blood were drawn simultaneously from the right and the left arm. Practically the same (normal) oxygen content was found in the two samples drawn after exercise as in the sample drawn during rest.

DISCUSSION.

The experiments bring out the fact that not every kind of exercise nor any amount with the legs affect the oxygen content of the venous blood drawn from a cubital vein. The difference between the results of the first four and that of the fifth experiment is probably caused by simply a difference in the amount of work done, which, of course, was much smaller in Experiment 5 than in the first four experiments. On the other hand, the first four experiments show the same effect after running up and down a flight of stairs once, as after running up and down five times, although the strain undoubtedly increased with the increased number of runnings. The difference between the results of the experiments with a bicycle (Experiments 6 and 7) and the first four experiments is striking. Whether this is due to the difference in the degree of the strain put on the subject, or whether it in some unknown way is associated with the special kind of work performed, we are unable to determine. It is generally acknowledged that climbing stairs is one of the most strenuous forms of exercise, and it is not improbable that it is a greater strain to run up and down stairs once, than to ride a bicycle for a period of 2 minutes. It was noted that the subject felt more tired after running up and down a flight of stairs once than after 2½ minutes on the bicycle (the resistance of which was increased considerably by means of a brake). On the other hand, climbing stairs did not increase the pulse or respiration rates more than the bicycle riding did. During the last experiment (Experiment 8), the conditions were fundamentally different from those during the other seven experiments, because in Experiment 8 the blood was not drawn from a resting region.

The effect on the venous blood drawn from a resting region is probably brought about when the strain reaches a certain high degree, and when it involves a sufficiently large number of muscles. And, in view of Lindhard's investigations on static work, which we have referred to in a previous paper, it seems not improbable that the effect takes place only when the oxygen supply of the working parts becomes insufficient. However, this is given only as a hypothetical explanation.

SUMMARY.

1. The previously described effect (a marked decrease in the oxygen content of venous blood drawn from the arm) of fast stair-running was just as marked if the subject ran up and down one flight of stairs once as it was after stair-running five times.

2. No change in the oxygen content of the venous blood drawn from the arm was observed (*a*) after the subject had walked slowly up and down one flight of stairs, (*b*) after riding a bicycle (with brakes on) for 2 to 2½ minutes, and (*c*) after exercise with the arm (lifting a heavy load).

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A NOTE ON THE EFFECT OF SOME ORGANIC ACIDS UPON THE URIC ACID EXCRETION OF MAN.

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Experiments by Lewis, Dunn, and Doisy (1918) seemed to indicate that the increased excretion of uric acid after the ingestion of amino-acids is not due either to specific dynamic action or to ammonia which has been split from the ingested amino-acid. Amino-acids (aspartic and glutamic) without specific dynamic action produce fully as great an increase in the uric acid eliminated as amino-acids which markedly stimulate the heat production. Neither ammonium chloride nor urea taken *per os* affect the excretion of uric acid. These possible explanations having been rendered unlikely, it occurred to us that the testing of the effect of the organic acids obtained by oxidative and by hydrolytic removal of the amino group might yield data of some interest.

The experiments were conducted on two normal men according to the same general scheme used by Lewis, Dunn, and Doisy. Most of the uric acid determinations in urine and all of those on blood and plasma were made by the Morris-Macleod (1922) method. We have found that this method yields very good results on plasma. To assure ourselves that our results were not due to interference with the colorimetric determination the uric acid has been isolated by the well known Kruger-Schmidt copper precipitation.[1] These values which agreed fairly well with the colorimetric values leave no doubt about the observations regarding the effects of the organic acids.

As the hydroxy- and keto-acids analogous to alanine are easily obtainable they were used in our experiments. It would have been

[1] We wish to express our thanks to Dr. Michael Somogyi for these determinations.

desirable to use other hydroxy- and keto-acids and with this in view we have performed two experiments with glycollic acid. Glyoxylic acid was not used because of the difficulty in obtaining it in a sufficiently pure condition and the very toxic effects of the substances which are frequently present as impurities. Approximately equimolecular quantities of the sodium salts of lactic acid and of pyruvic acid were ingested after the collection of two or three normal hourly specimens. The effects of both acids were very striking. Pyruvic acid increased the hourly excretion of uric acid from 17 to 25 mg. in one experiment and in the other from 16 to 28 mg. which was about the same increase as that produced by an equal amount of alanine. Both lactic and glycollic acids, on the contrary, caused an immediate depression in the quantity of uric acid excreted. In all of these experiments there was an abrupt fall to about one-half the quantity excreted prior to the ingestion of the acid. Data which are given in Tables I and II illustrate the contrast in the effects of these compounds on the excretion of uric acid.

The explanation of these experiments is very difficult and our data are insufficient to decide which of the various possibilities may be correct. Lewis, Dunn, and Doisy referred the increased excretion following amino-acids to an increased production (stimulation of endogenous metabolism) such as occurs when the subject passes from a low to a high protein diet. In so far as our data go this theory is not shaken. In Table III, we have collected the results of our various experiments on the uric acid of blood or plasma. It is evident that we have found some slight variations in two samples of blood drawn from the same normal fasting subject at a 2 or 3 hour interval. About the same variation prevails when a pyruvic or an amino-acid is ingested, but the variation after lactic acid seems somewhat more pronounced. There appears to be a slight rise in the uric acid in the plasma after the ingestion of lactic acid which, coupled with the decreased excretion, would lead one to think of an elevated threshold in the kidney.

The changes in plasma uric acid are, to be sure, very small and it is possible that even such minute changes as would escape detection by our analyses could cause decided alterations of hourly urinary uric acid. But granting this, it still seems that

TABLE I.

Uric Acid in Urine in Milligrams per Hour.

Normal human subject. Male instructor. Weight 63 kilos.

Hour.	Normal.	Alanine.	Pyruvic acid.	Lactic acid.	Glycollic acid.
a.m.					
7–8	20.8	18.6*	17.3	20.6	22.5
8–9	20.0	20.9	17.1†	21.0‡	23.6§
9–10	17.8	29.5	20.9	12.2	14.3
10–11	19.1	27.5	24.0	6.0	11.8
11–12	19.2	21.1	25.2	6.0	11.0
p.m.					
12–1	19.0	16.9	21.8	10.2	13.4
1–2	19.0	17.4	16.8	14.8	16.1
2–3	17.2		16.0	22.9	20.4
3–4	18.2		14.0	22.9	

* 21.5 gm. of alanine taken at 8.00 a.m.

† 16 gm. of pyruvic acid taken at 9.00 a.m.

‡ 12 gm. of lactic acid taken at 9.10 a.m.

§ 10 gm. of glycollic acid taken at 9.00 a.m.

No food after supper of previous evening until completion of experiment.

TABLE II.

Uric Acid in Urine in Milligrams per Hour.

Male. Medical student. Weight 59 kilos.

Hour.	Normal.	Pyruvic acid.	Lactic acid.	Gycollic acid.
a.m.				
7–8	17.1	16.5		
8–9	17.3	18.5*	15.7	22.5
9–10	18.0	18.9	16.6	21.6
10–11	18.2	26.7	16.7†	22.8‡
11–12	17.7	28.3	15.1	12.4
p.m.				
12–1	14.3	26.7	8.4	8.8
1–2	13.6	20.3	8.4	10.0
2–3	13.3	15.1	11.2	10.5
3–4	11.5	12.5	14.4	11.2

* 16 gm. of pyruvic acid taken at 9.20 a.m.

† 14 gm. of lactic acid taken at 10.30 a.m.

‡ 10 gm. of glycollic acid taken at 10.45 a.m.

No food after supper of previous evening until completion of experiment.

lactic acid causes a slight rise in the plasma which is concomitant with the decrease in urinary uric acid and we are inclined to the view that these results are due to an increased threshold of the kidney for uric acid.

TABLE III.

Blood and Plasma Uric Acid in Milligrams of Uric Acid per 100 Cc.

	Normal.	Amino-acid.	Pyruvic acid.	Lactic acid.
Sample 1	3.6	3.4	3.8	3.7
" 2	3.5	3.4	3.8	3.9
Increase	−0.1	0.0	0.0	+0.2
Sample 1	4.2	3.3		4.2
" 2	4.2	3.3		4.6
Increase	0.0	0.0		+0.4
Sample 1	5.5			4.6
" 2	5.5			4.9
Increase	0.0			+0.3
Sample 1	3.8			
" 2	3.9			
Increase	+0.1			
Sample 1	4.6	4.8	4.8	4.5
" 2	5.0	4.8	4.8	4.8
Increase	+0.4	0.0	0.0	+0.3
Sample 1	4.5			
" 2	4.5			
Increase	0.0			
Sample 1	4.7			
" 2	4.9			
Increase	+0.2			
Average change	+0.1	±0.0	±0.0	+0.3

The first sample of blood was generally taken a few minutes before the organic acid and the second sample 2 or 3 hours later.

It seemed that the effect of lactic acid upon the well known increase of urinary uric acid which occurs after eating cottage-cheese or sweetbreads would be of some interest. The data of Table IV show that instead of the usual increase of uric acid

excretion which occurs after the ingestion of these protein foods a decreased output which is much below the normal is obtained. From these experiments we draw the inference that lactic acid affects the elimination of uric acid whether it be from exogenous or endogenous sources. Of course, our experiments do not exclude an alteration of the rate of conversion of purines to uric acid, but since we have found a slight rise in the plasma and have failed to find an increase of non-uric acid purine nitrogen in the

TABLE IV.

Effect of Lactic Acid upon Excretion of Endogenous and Exogenous Uric Acid.

Hour.	Cottage-cheese.	Cottage-cheese and lactic acid.	Thymus.	Thymus and lactic acid.
1st	15.6	15.9	**21.3***	**21.3***
2nd	**18.5**†	**17.7**‡	25.0	**17.0**§
3rd	26.5	19.4	36.9	6.7
4th	30.3	**22.4**	44.4	14.5
5th	33.3	21.3¶	44.4	32.4
6th	33.3	9.4	35.7	33.3
7th	30.3	6.9	34.8	32.0
8th	29.4	12.9	35.0	30.7
9th		18.2	25.5	30.6
10th			23.1	29.5

* 200 gm. of thymus.

† 480 gm. of cottage-cheese.

‡ 500 gm. of cottage-cheese.

§ 10 gm. of lactic acid as sodium salt.

¶ 14 gm. of lactic acid as sodium salt.

Several experiments of this type, in which 5 gm. of sodium benzoate were taken instead of the larger dose of sodium lactate, yielded results which were practically identical with these data. Sodium hippurate appeared to have no influence upon the quantity of uric acid eliminated.

urine we are inclined to view the phenomenon as an effect upon the kidney.

From the results of these experiments two logical questions arise: (1) May the elimination of uric acid during and following exercise (Kennaway, 1909) be influenced by the production of lactic acid? (2) May the opposite effects of lactic and pyruvic or amino-acids upon the uric acid excretion be taken as evidence against the hydrolytic deamination hypothesis? With regard

to the first question, we have conducted some preliminary experiments which we are extending before reporting their results. As to the second question, we may say that there is a rapid rise (unpublished observations) in the excretion of nitrogen after the ingestion of amino-acids which we consider to be due to deamination. The deaminized product is rapidly made available and if much of it were lactic acid it is probable that a decreased output of uric acid would result. Actually, an increase occurs so it may be considered probable that hydrolytic deamination is not the chief pathway of decomposition; oxidative deamination is not so excluded.

SUMMARY.

The ingestion of sodium salts of lactic acid causes a decrease, and of pyruvic acid an increase in the excretion of uric acid.

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COMPOSITION OF CORN POLLEN.

II. CONCERNING CERTAIN LIPOIDS, A HYDROCARBON, AND PHYTOSTEROL OCCURRING IN THE POLLEN OF WHITE FLINT CORN.

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INTRODUCTION.

Continuing the investigation of the constituents contained in corn pollen, we report at this time a study of certain lipoids, particularly the unsaponifiable constituents in the fat and ether extract of corn pollen. The pollen was gathered in 1920 from White Flint corn of the variety known as Luces Favorite.

We had hoped to isolate and study the phosphatide contained in this pollen, but it was impossible to accomplish much in this direction because the loss of material in the process of purification was so great that the quantity of the final phosphatide was too small to permit of any extended investigation.

It was mentioned in the first paper (1) that the alcoholic extract of pollen deposited, on standing in the ice box, a small amount of colorless crystals, "Substance A," which after crystallizing from alcohol melted at 88–89°C., but which, owing to lack of material, could not be identified. In the present investigation we obtained a larger amount of this substance and it was identified as phytosterol palmitate.

Associated with the crude phytosterol palmitate was a small percentage of a saturated hydrocarbon which melted at 63–64°C. It is apparently identical with the normal nonakosane, $C_{29}H_{60}$, which was isolated from Indian hemp by Wood, Spivey, and Easterfield (2).

Dry corn pollen when extracted with absolute ether yields about 1.5 per cent of ether extract as shown in the first paper (1). This ether extract was found to contain 14 per cent of a saturated hydrocarbon identical with the one mentioned above. On saponification of the ether extract we obtained 4.4 per cent of pure phytosterol.

In the preparation of the phosphatide there remained in the acetone mother liquors a large amount of fat which, on evaporation of the solvent, was obtained as a dark colored, thick oil. On saponifying this fat we obtained 25 per cent unsaponifiable material which consisted of a mixture of various phytosterols.

The phytosterol preparations obtained from this fat gave the usual color reactions for phytosterol and in composition they agreed closely with the accepted formula $C_{27}H_{45}OH$. They showed, however, several marked differences in properties from the ordinary phytosterol. The preparations which we obtained were optically inactive, they crystallized without any water of crystallization, and none had the same melting point as ordinary phytosterol. Bromine derivatives could not be obtained in a pure state because there was not only addition of bromine but also substitution with liberation of hydrobromic acid, and the resulting bromine derivatives were non-crystalline and turned very dark in color. The purified phytosterol preparations were subject to spontaneous decomposition, possibly due to oxidation, with production of a yellow color, and there developed a pronounced rancid odor.

All the various phytosterol preparations, isolated from the pollen, differed in melting points, and the melting points of their acetyl derivatives ranged from 101–134°C.

EXPERIMENTAL.

The pollen was dried to constant weight at room temperature *in vacuo* over sulfuric acid. The dried pollen, 1,422 gm., was digested with frequent shaking in 2.5 liters of absolute alcohol at a temperature of 60°C. The hot mixture was filtered on a large Buchner funnel and washed with 1 liter of hot absolute alcohol. The residue was again extracted three times in the same manner. The extract obtained measured about 10 liters

and it was perfectly clear. It was of slightly brownish yellow color, and it showed a faint acid reaction to litmus.

This treatment removed practically all the phosphatide. Exhaustive extraction of the pollen residue with ether or chloroform in Soxhlet apparatus gave only very small amounts of extractive which contained merely traces of phosphorus.

The alcoholic extract was allowed to stand at 0°C. for 2 days. A considerable amount of crystalline material separated slowly. This substance was filtered on a Buchner funnel, washed with a little ice-cold alcohol, and dried in vacuum over sulfuric acid. The filtrate was reserved for further examination.

Examination of the Above Crystalline Substance.

The dry substance was slightly yellowish in color and it weighed 22 gm. It was practically insoluble in cold alcohol and only slightly soluble in hot alcohol or in ether. It was partly soluble in water. In acetone and other organic solvents it was only slightly soluble. After decomposing by the Neumann method it gave a slight reaction for phosphoric acid, indicating that it contained a small amount of phosphatide. It reduced Fehling's solution on boiling, indicating an admixture of reducing sugar.

The substance was digested with warm ether but only a small amount appeared to go into solution. The insoluble residue was treated as will be mentioned later under "Substance A."

The ethereal solution on evaporation left a yellowish oily liquid which solidified on cooling. Digesting it in cold acetone removed the yellow coloring matter and left a nearly white powder. After filtering, washing with acetone, and drying, the substance weighed 4.1 gm.

On evaporating the acetone washings there remained 1.2 gm. of a yellow wax-like material which was not further examined.

The white substance mentioned above was very slightly soluble in cold alcohol. When heated in absolute alcohol, it liquefied, forming small oily drops which dissolved slowly on continued boiling. On cooling it separated partly as voluminous flakes and partly in the form of burr-shaped aggregates of thin plates, and it evidently consisted of at least two substances.

A separation was affected by allowing its solution in absolute alcohol to cool very slowly and by not allowing the temperature

to fall below 50°C. Burr-shaped aggregates of thin plates crystallized out from the hot solution, and these were filtered and washed with warm alcohol.

The filtrate was saved and examined as will be discussed later under "Substance B."

The crystalline substance was again recrystallized from warm alcohol in the same manner as above and finally recrystallized many times from absolute alcohol. In the purification of this substance the mother liquors were saved and on concentration yielded "Fraction C."

The substance was finally obtained as snow-white crystals which separated, on slowly cooling the absolute alcohol, in aggregates of long thin plates. The substance weighed 1.5 gm. and it melted at 88–88.5°C. (uncorrected). It gave the Liebermann-Burchard reaction, and it appeared to be identical with "Substance A" mentioned in the first paper (1).

For analysis the substance was dried in vacuum over phosphorus pentoxide at the temperature of boiling chloroform, but there was no loss in weight.

0.1063 gm. substance: 0.1162 gm. H_2O and 0.3228 gm. CO_2.
Found. C 82.82, H 12.23 per cent.

This substance is evidently phytosterol palmitate. Ritter (3) described a synthetic phytosterol palmitate which melted at 90°C.

Calculated for $C_{43}H_{76}O_2$ = 624.
C 82.69, H 12.18 per cent.

The substance was optically inactive.

Saponification of the Phytosterol Palmitate.

1 gm. of the substance was saponified by boiling for 2 hours with 65 cc. of alcoholic potassium hydroxide. The phytosterol was isolated in the usual way (4), again boiled with alcoholic potassium hydroxide for $\frac{1}{2}$ hour, and purified by repeated crystallization from aqueous alcohol. About 0.4 gm. of snow-white thin plate-shaped crystals was finally obtained. The substance gave the usual color reactions for phytosterol, but it did not show a definite melting point. It softened at 125°, but it was not

completely melted until heated to 128°C. By repeated fractional crystallizations it was eventually separated into two parts. A smaller portion (A) which melted at 122°C. (uncorrected) separated first and a fraction (B) which melted at 136.5°C. (uncorrected) was obtained from the mother liquors. Both fractions gave similar color reactions, and there was very little difference in composition as determined by analysis. Both fractions separated from aqueous alcohol without any water of crystallization, and they did not lose in weight on drying at 105°C. in high vacuum over phosphorus pentoxide.

The following results were obtained on analysis.

Preparation A. Found. C 83.20, H 11.73 per cent.
Melting at 122°C.

Preparation B. Found. C 83.80, H 11.73 per cent.
Melting at 136.5°C.
Calculated for phytosterol, $C_{27}H_{45}OH$ = 386.
C 83.93, H 11.91 per cent.

Preparation of the Acetyl Derivative.

0.1 gm. of Preparation B, melting at 136.5°C., was boiled with acetic anhydride, and the excess of the latter distilled off in vacuum. The residue was very soluble in ethyl alcohol and it was therefore recrystallized several times from methyl alcohol in which it was very slightly soluble. It was obtained in the form of snow-white, rather large, needle-shaped crystals which melted at 101°C. (uncorrected). Further recrystallization did not alter the melting point.

It is evident from the low melting point of the acetyl derivative that this phytosterol, although it melted at 136.5°C., differed from the usual phytosterol or sitosterol which gives an acetate, melting at 127°C.

Unfortunately, there was not a sufficient amount of Preparation A available for preparing an acetyl derivative.

Isolation of Palmitic Acid.

After removing the phytosterol from the saponification mixture the fatty acid was obtained on acidifying and shaking out with ether. After washing and drying the ethereal solution it was

evaporated, leaving a practically colorless residue. After crystallizing twice from alcohol, it was obtained as snow-white crystals.

The substance melted at 62.5°C. (uncorrected) and began to solidify at 58°C. For analysis the substance was dried in high vacuum at the temperature of boiling chloroform, but it did not lose in weight.

0.1009 gm. substance: 0.1149 gm. H_2O and 0.2781 gm. CO_2.

Found. C 75.17, H 12.74 per cent.

For palmitic acid, $C_{16}H_{32}O_2$ = 256.

Calculated. C 75.00, H 12.50 per cent.

Examination of Substance "C" Obtained from the Mother Liquors on Recrystallizing the Above Phytosterol Palmitate.

On evaporating the alcoholic mother liquors a colorless, crystalline residue was obtained. This was recrystallized several times from absolute alcohol and then saponified with alcoholic potassium hydroxide. The fatty acid could not be identified. It differed from palmitic acid. It was very soluble in alcohol and it could not be obtained in crystalline form.

The higher alcohol was isolated in the same manner as phytosterol and was purified by crystallizing many times from absolute alcohol. The substance was snow-white in color and separated in very thin plate-shaped crystals. The substance did not contain any water of crystallization, and it melted at 136°C. (uncorrected). It did not absorb bromine, and it did not give any of the phytosterol color reactions. On analysis the following result was obtained:

Found. C 82 32, H 14.08 per cent.

The percentage composition agrees with that of a saturated alcohol having the formula $C_{30}H_{62}O$, which corresponds to the formula of myricyl alcohol; but it could not be myricyl alcohol, because the melting point was much too high.

Examination of the Filtrates Containing Substance "B".

As mentioned above, the phytosterol palmitate was separated from the mixture by allowing it to crystallize very slowly at a

temperature of 50°C. The mother liquors were concentrated and allowed to cool. The substance separated in very thin, snow-white plates. This was repeatedly recrystallized from absolute alcohol, but it did not show a very definite melting point. It softened at 58° and liquefied at 64° to a not wholly clear liquid.

The substance was now boiled with 100 cc. of alcoholic potassium hydroxide for 3 hours. The solution was diluted with water and extracted with ether. The ethereal solution was washed three times with water, filtered, and the ether evaporated. The substance was only slightly soluble in alcohol and it was therefore taken up in warm ether and diluted with 10 cc. of alcohol. The ether was allowed to evaporate spontaneously at room temperature, when the substance separated in colorless rather large, very thin, plate-shaped crystals. It was recrystallized from 120 cc. of absolute alcohol. The substance melted in the hot alcohol, forming small oily drops which dissolved slowly on continued boiling. On cooling it separated as fine silky needles which entirely filled the liquid. It was filtered, washed in absolute alcohol, and dried in vacuum over sulfuric acid. It weighed 0.8 gm. Heated in a capillary tube it melted at 63.5–64°. It was again twice recrystallized in the same manner, but the melting point did not change.

The substance was very slightly soluble in ethyl and methyl alcohol and not very soluble in ether, but in chloroform it was readily soluble. It was easily soluble in warm benzene, but on cooling it crystallized out again.

For analysis the substance was dried in vacuum at the temperature of boiling chloroform, but it did not lose in weight.

0.0948 gm substance: 0.1264 gm. H_2O and 0.2960 gm. CO_2.

Found. C 85.15, H 14.92 per cent.

Owing to its slight solubility in cold benzene and its insolubility in other suitable solvents, the freezing point method could not be used for determining its molecular weight. The less satisfactory boiling point method, using benzene as solvent, was tried. The rise in the boiling point corresponded to a molecular weight of 392.

The substance did not absorb bromine, and it was not affected by hot or cold concentrated sulfuric acid or by boiling with a mixture of sulfuric and nitric acid.

The composition and properties indicate that the substance was a saturated hydrocarbon. It is probable that it is identical with the normal nonakosane, $C_{29}H_{60}$, isolated by Wood, Spivey, and Easterfield (2), from Indian hemp which melted at 63.5–64°C.

For $C_{29}H_{60}$ = 408. Calculated. C 85.29, H 14.70 per cent.

Examination of the Ether-Insoluble Material "A".

The portion of the original substance which was insoluble in ether was treated with water in order to remove soluble carbohydrates, etc. It formed a permanent emulsion with water, but on the addition of acetone a good separation was obtained. The mixture was centrifuged; and the solid material was again stirred up with water, separated by acetone, and again centrifuged. This treatment was repeated a third time. The substance was now nearly pure white in color. After filtering, washing in acetone, and drying in vacuum over sulfuric acid, it weighed 4.2 gm. After decomposing by the Neumann method, a considerable reaction for phosphoric acid was obtained indicating an admixture of phosphatide. The phosphorus-containing substance was subsequently removed by the treatment with acetic ester.

The substance was only slightly soluble in the usual organic solvents, and it showed very little tendency to crystallize. It separated in amorphous flakes from ethyl or methyl alcohol. From acetic ester it separated as a powder without definite crystalline structure.

The material was dissolved in 300 cc. of boiling acetic ester and filtered from a small amount of insoluble matter. The nearly white powder which separated on cooling was filtered and the above treatment with boiling acetic ester was repeated. The substance now separated in practically colorless fine needles which, after filtering, washing with acetic ester, and drying in vacuum over sulfuric acid, weighed 2.9 gm.

The residue which was insoluble in acetic ester gave a heavy reaction for phosphorus after decomposing by the Neumann method.

The substance purified as mentioned above was free from nitrogen, phosphorus, sulfur, and halogens. In chloroform solution it immediately decolorized bromine without liberation of hydro-

bromic acid, and it gave the Liebermann-Burchard reaction for phytosterol. Its melting point was not sharp. It began to soften at 85°, but it was not completely melted until heated to 90°C. These reactions and melting point indicated that the substance was similar to the phytosterol palmitate previously isolated.

Without further purification the substance was saponified, and the products of the saponification were separated and purified.

The fatty acid was isolated as described before, and it was purified by repeated crystallization from methyl alcohol until it was pure white in color. Its properties and composition corresponded to palmitic acid. It melted at 62.5°C. (uncorrected), and the melting point did not change on further crystallizations. On analysis the following result was obtained:

Found. C 75.18, H 12.62 per cent.
For palmitic acid, $C_{16}H_{32}O_2$ = 256.
Calculated. C 75.00, H 12.50 per cent.

The phytosterol was again separated into two portions by repeated fractional crystallizations. The lower melting fraction began to soften at 120° and melted at 123°C., but in spite of numerous recrystallizations it was impossible to obtain a preparation showing a sharp or definite melting point.

The higher melting fraction melted at 136.5°C. (uncorrected) which did not change on further recrystallization. The substance crystallized in beautiful long, thin, colorless plates without any water of crystallization. On drying at 105° in high vacuum it did not lose in weight. The following result was obtained on analysis:

0.1039 gm. substance: 0.1087 gm. H_2O and 0.3212 gm. CO_2.
Found. C 84.31, H 11.70 per cent.
For $C_{27}H_{45}OH$ = 386. Calculated. C 83.94, H 11.91 per cent.

The acetyl derivative was prepared and purified by crystallization from methyl alcohol. Its melting point was constant at 101°C. (uncorrected).

It is curious that the phytosterol preparations which we obtained had the same melting point as the phytosterol described as sitosterol (5), and yet they gave acetyl derivatives which had a very much lower melting point than sitosterol acetate which

melts at 127°C. The low melting point of the acetyl derivatives and the fact that the preparations did not contain any water of crystallization indicates a decided difference from the usual phytosterol. Unfortunately, there was not a sufficient quantity of the purified material available to permit the determination of optical properties, but the phytosterol palmitate was inactive. It is likely, therefore, that the phytosterol itself was inactive.

Examination of the Alcoholic Extract of Pollen.

Isolation of the Phosphatide.

The alcoholic extract, after filtering off the crystalline precipitate referred to above, was concentrated under reduced pressure at a temperature of 40°C., to about 2.5 liters. Further concentration in vacuum was impossible owing to excessive foaming. When two-thirds of the alcohol had been distilled off, the solution turned cloudy and there separated a considerable quantity of a yellowish, gummy material which was sparingly soluble in alcohol, insoluble in ether, but readily soluble in water. The addition of ether to the concentrated alcoholic solution caused a voluminous, white, amorphous precipitate. In order to remove this ether-insoluble material, the alcoholic solution was precipitated by adding about 1.5 volumes of ether. After standing over night, the precipitate had settled to a compact mass on the bottom of the container. The ethereal solution was decanted and the residue rinsed several times with ether.

The ether-insoluble substance was a dark brown-colored, gummy, rather sticky material. It was soluble in hot alcohol and readily soluble in water. After drying in vacuum over sulfuric acid, it weighed 108 gm. The substance contained small quantities of nitrogen and phosphorus, but the greater portion evidently consisted of sugar. It was reserved for special examination.

The ether-alcohol solution was evaporated under reduced pressure to a thick syrup. This syrup was heated with ether which dissolved the greater portion. After the ethereal solution had settled, the clear liquid was decanted and the residue washed with ether. The ethereal solution was then concentrated on the water bath to a syrup. This was taken up in ether, and a small

amount of insoluble matter was separated by decantation. The phosphatide was now separated from the ethereal solution by precipitating with acetone. It was taken up in ether and reprecipitated with acetone. These operations were repeated until the phosphatide was entirely soluble in ether forming a practically clear solution. The crude phosphatide obtained in this manner was yellowish light brown in color and of salve-like consistency when freshly precipitated. After drying in vacuum over sulfuric acid, it weighed 11.6 gm. The dry product was hard and brittle and could be powdered, but it was slightly hygroscopic. After hydrolyzing a small sample of this substance by boiling with dilute sulfuric acid, it gave a heavy reaction for sugar with Fehling's solution (6). The crude phosphatide was further purified by the method outlined by Maclean (7). During this process most of the material disappeared, and there finally remained only 1 gm. of a nearly white, amorphous preparation. After decomposing by the Neumann method this substance was found to contain 4.09 per cent of phosphorus. In view of the small amount of this substance which was obtained, further experiments were not made. The phosphorus content, however, indicates that it was a typical lecithin.

Examination of the Fat from Corn Pollen.

A large amount of fat was contained in the acetone mother liquors after precipitating the phosphatide. The fat was obtained on evaporating the acetone and drying the residue in vacuum over sulfuric acid. It formed a dark brown, oily substance which after standing for several weeks at room temperature, deposited a considerable amount of plate-shaped crystals in star-like aggregates. The oil weighed 110 gm. The substance contained a trace of phosphorus, but no sulfur. The iodine number, determined by the Hanus method (8), was found to be 186.5.

Saponification of the Fat from Corn Pollen.

We desired to obtain some phytosterol from this fat in order to compare its properties with those of the preparations isolated from the phytosterol palmitate. For this purpose 100 gm. of

the fat were saponified with alcoholic potassium hydroxide and the unsaponifiable material was isolated in the usual way. In order to obtain all of the unsaponifiable material from the soap solution it was necessary to extract it six times with ether.

The crude, yellowish colored residue which remained on evaporating the ether was again boiled with alcohol potassium hydroxide and on cooling a large part of it separated in colorless, plate-shaped crystals. This was filtered and washed with dilute alcohol and water until free from alkali. The substance was practically snow-white and after drying in vacuum over sulfuric acid it weighed 19.5 gm. The balance of the unsaponifiable material was obtained from the filtrate by extraction with ether. Unfortunately, a small portion of this solution was lost through an accident. After evaporating the ether and drying the residue in vacuum over sulfuric acid, it weighed 5.40 gm. A part of this material consisted of a yellowish oil of pleasant aromatic odor which adhered to the crystals.

The total unsaponifiable material recovered was 24.90 gm., but in view of the fact that some had been lost, it is evident that the fat from corn pollen contained somewhat more than 25 per cent of unsaponifiable matter.

Purification and Properties of the Phytosterol from the Fat of Corn Pollen.

The first crystalline preparation, 19.5 gm., mentioned above was recrystallized three times from 95 per cent alcohol. The product was snow-white and consisted of thin plate-like crystals generally arranged in round balls. The purified material weighed 11.25 gm. It gave the Liebermann-Burchard reaction and readily absorbed bromine in chloroform solution. The substance was optically inactive. Heated in a capillary tube it began to soften at 140°, and melted to a clear fluid at 143°C. (uncorrected).

On concentrating the mother liquors from the above and recrystallizing the residue 5.0 gm. of snow-white, thin, plate-shaped crystals were obtained which softened at 120° and melted to a clear fluid at 124°C. (uncorrected).

The first product, which melted at 143°C., was again recrystallized three times from 95 per cent alcohol. It crystallized in the same form as before. The substance now weighed 7.7 gm.

It again softened at 140° and melted at 143°C. (uncorrected). It did not lose in weight on drying for 3 hours in high vacuum at 105°C., over phosphorus pentoxide. On analysis the following result was obtained.

0.1299 gm. substance: 0.1372 gm. H_2O and 0.3998 gm. CO_2.
Found. C 83.93, H 11.81 per cent.
For $C_{27}H_{45}OH$ = 386. Calculated. C 83.93, H 11.91 per cent.

The material contained in the mother liquors from the above crystallizations was recovered upon concentration of the solvents in vacuum. The melting point varied from 130–134°C.

The various fractions of the lower melting phytosterol which had been recovered from the mother liquors were united and fractionally crystallized many times. It was impossible, however, to obtain any product showing a constant melting point. The lowest melting fraction softened at 116° and became fluid at 121°C., and the highest softened at 132° and became fluid at 135°C. A number of fractions were obtained which melted between the above limits.

Heyl (9) has recently described some phytosterols obtained from ragweed pollen which could be separated only with great difficulty. This author also reports a trace of a hydrocarbon in the unsaponifiable material.

Acetyl Derivative of the Foregoing Phytosterol Preparation.

The purified phytosterol as analyzed above, 5 gm., was boiled with 100 cc. of acetic anhydride for about 2 hours. On cooling, the greater portion crystallized out in large, irregular, plate-shaped crystals. After standing in a freezing mixture for some time the crystals were filtered and washed in cold glacial acetic acid and finally in water. It was dried in vacuum over sulfuric acid, and it weighed 4.2 gm.

The filtrate was diluted with water when a further quantity of material separated which was filtered, washed with water, and dried as above. It weighed 1.2 gm. The total yield was 5.4 gm.; calculated 5.5 gm.

The first crystalline product was recrystallized from 95 per cent. alcohol from which it separated in square plates. It was snow-white in color. The substance began to soften at 110°C.

and melted at 114°C. (uncorrected). It was again recrystallized, but the melting point did not change. The substance did not lose in weight on drying at 105° in vacuum over phosphorus pentoxide. On analysis the following result was obtained:

0.1590 gm. substance: 0.1588 gm. H_2O and 0.4758 gm. CO_2.
Found. C 81.61, H 11.17 per cent.
For phytosterol acetate, $C_{29}H_{48}O_2$ = 428.
Calculated. C 81.31, H 11.21 per cent.

The composition of the above phytosterol and its acetyl derivative agree closely with the theory. The melting point, however, approaches that of cholesterol and cholesterol acetate. But the substance differs from the usual phytosterol, as well as from cholesterol, in that it is optically inactive, and it does not contain any water of crystallization.

Bromination of the Acetyl Derivative.

An attempt was made to brominate the above acetyl derivative by the method of Windaus and Hauth (10). 3.6 gm. of the acetyl derivative were dissolved in 36 cc. of ether and a solution was added containing 2.25 gm. of bromine in 45 cc. of glacial acetic acid. The bromine was absorbed immediately, and there was some rise in temperature. Some hydrobromic acid was given off; and the solution, which was colorless at first, turned pale green and after a few minutes very dark green in color. More bromine was added in portions of 1.5 gm. dissolved in 20 cc. of glacial acetic acid until a total of 7.75 gm. of bromine had been added. The color was so dark that it was almost impossible to determine when the solution contained an excess of bromine. Much hydrobromic acid was given off. Nothing crystallized from this solution, even after standing for some time. The mixture was, therefore, diluted with water and extracted with ether. The ethereal solution was washed with dilute alkali and water, filtered, and the ether evaporated. The residue was nearly black in color, and it could not be crystallized. After drying in vacuum over sulfuric acid, it weighed about 6 gm. This would represent an addition of 3 atoms of bromine. The substance was readily soluble in ether and only slightly soluble in alcohol. From hot alcohol it separated on cooling in amor-

phous flakes. It was found to be impossible to decolorize the alcoholic or ethereal solutions, and the substance could not be obtained in crystalline form. It is evident, therefore, that the substance did not contain any stigmasterol (11).

Further Purification of the Phytosterol from Corn Pollen Fat.

The phytosterol described above which melted at 143°C. was, as previously mentioned, obtained in snow-white, thin, plate-shaped crystals. The substance, which remained after preparing the acetyl derivative, on standing for a few days in the laboratory in a well stoppered glass bottle turned yellowish in color and developed a distinct, somewhat rancid, odor. This material was then further recrystallized from methyl alcohol. There was a steady rise in the melting point after each crystallization until the melting point remained constant at 154°C. (uncorrected). It separated from methyl alcohol in pure white, rather large rosettes, consisting of fine needle-shaped crystals. This purified substance was also optically inactive. It lost no weight on drying at 105°C. in high vacuum over phosphorus pentoxide. On analysis the following result was obtained:

0.1053 gm. substance: 0.1128 gm. H_2O and 0.3229 gm. CO_2.
Found. C 83.63, H 11.98 per cent.

The recrystallizations from methyl alcohol had raised the melting point by 11°, from 143°C. to 154°C., but there was no change in composition that could be detected by analysis.

The acetyl derivative was prepared in the usual way and the product recrystallized from methyl alcohol. It separated in large, very thin, plate-shaped crystals. The dry substance was snow-white in color. This melted at 134°C. (uncorrected).

Examination of the Unsaponifiable Matter Contained in the Ether Extract of Corn Pollen.

The ether extract obtained from 590 gm. of corn pollen weighed 9 gm. after drying in vacuum over sulfuric acid. It was of a dirty dark green color and soft, wax-like consistency. It was saponified by boiling with 200 cc. of alcoholic potassium hydroxide for 3 hours.

Substance 1.—The hot alkaline solution was filtered to remove a small quantity of an insoluble oily substance. On cooling, this oil solidified, forming a white solid. This substance was recrystallized three times from absolute alcohol and was obtained in the form of snow-white, thin, plate-shaped crystals which weighed 0.3 gm. The substance did not give the Liebermann-Burchard reaction, and it did not absorb bromine in chloroform solution. Heated in a capillary tube it melted at 63°C. (uncorrected). It did not lose in weight on drying at the temperature of boiling chloroform in vacuum over phosphorus pentoxide. On analysis the following result was obtained:

Found. C 85.05, H 14.98 per cent.

Its properties and composition correspond to those previously found for the saturated hydrocarbon $C_{29}H_{60}$.

Substance 2.—On cooling the filtrate, after removing the above oily substance, there separated a small quantity of colorless, plate-shaped crystals. These were filtered off, washed in a little cold alcohol, and dried in vacuum over sulfuric acid. They weighed 0.95 gm. The substance was recrystallized five times from absolute alcohol and was obtained in snow-white, thin, plate-shaped crystals. It did not absorb bromine in chloroform solution, and it did not give the Liebermann-Burchard reaction. It melted at 63°C. (uncorrected). It was again recrystallized, and the melting point did not change. The following result was obtained on analysis:

Found. C 85.03, H 15.22 per cent.

The composition and properties agree with those observed with "Substance 1" above and correspond to the saturated hydrocarbon $C_{29}H_{60}$.

The total amount of this hydrocarbon obtained from 9 gm. of ether extract was 1.25 gm. which is equal to 14 per cent.

Substance 3.—The balance of the unsaponifiable material was extracted with ether after the alcohol had been partly evaporated and the liquid diluted with water. After purifying in the usual manner and recrystallizing five times from methyl alcohol, the substance was obtained as snow-white, long, thin, plate-shaped crystals which gave the usual reactions for phytosterol. It

weighed 0.4 gm. The ether extract contained, therefore, not less than 4.4 per cent of phytosterol. The substance melted at 123°C. After it had been further recrystallized about twenty times from methyl alcohol, the melting point remained constant at 125–126°C. (uncorrected). The small quantity of material available prevented any further purification, and we are unable to state whether the substance was homogeneous or not.

SUMMARY.

The following substances have been isolated from the alcoholic and ether extracts of corn pollen.

1. Phytosterol palmitate melting at 88–88.5°C. On saponification this substance yielded: (*a*) palmitic acid melting at 62.5°C.; (*b*) one fraction of phytosterol melting at 122°C.; and (*c*) another fraction of phytosterol which melted at 136.5°C. The acetyl derivative of the latter melted at 101°C.

2. A saturated hydrocarbon which melted at 63–64°C. This substance is evidently identical with the normal nonakosane, $C_{29}H_{60}$.

3. A saturated alcohol, $C_{30}H_{62}O$, which melted at 136°C. and which has not been identified.

4. A phosphatide which contained 4.09 per cent of phosphorus.

5. The fat extracted from corn pollen with absolute alcohol contained 25 per cent of unsaponifiable matter consisting of a mixture of phytosterols. The melting points of these phytosterol fractions ranged from 121–154°C. The acetyl derivative of the latter melted at 134°C.

6. The ether extract contained 14 per cent of the saturated hydrocarbon, $C_{29}H_{60}$, melting point 63°C., and 4.4 per cent of phytosterol melting at 125–126°C.

The phytosterol preparations isolated from corn pollen differed from ordinary phytosterol in several particulars: (*a*) They were all free from water of crystallization. (*b*) They were optically inactive. (*c*) The melting points of these phytosterols as well as of their acetyl derivatives all differed from those of ordinary phytosterol.

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THE RATE OF UREA EXCRETION.

VII. THE EFFECT OF VARIOUS OTHER FACTORS THAN BLOOD UREA CONCENTRATION ON THE RATE OF UREA EXCRETION.*

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We have shown that under certain special conditions the rate of urea excretion varies within narrow limits in direct proportion with the blood urea concentration. In any one individual, therefore, the ratio: $\frac{\text{urea in 1 hour's urine}}{\text{urea in 100 cc. of blood}}$ is under these conditions a constant (1). If now these conditions are all complied with, but in addition some new factor is introduced, the effect on the rate of urea excretion can be measured by the deviation of the ratio from its constant value. Most of the experiments given in this paper were carried out on the two subjects, Add. and Dru., whose ratios under standard conditions are given in Paper V of this series (1). For Add. the average ratio was 46.1 with a variability of ±6.9 per cent, and for Dru. the average was 56.2 with a variability of ±4.8 per cent. But some of our experiments on Dru. were carried out under conditions which differed from those we have called "standard" in that no urea was given. In these experiments the blood urea concentration was at a low level so that any error in the technique of the blood urea determinations would have a relatively greater effect on the ratio than in the experiments in which the blood urea concentration had been increased by urea administration. The method was, therefore, reexamined and a modification, used only in these experiments, was introduced which probably

* This work was aided by a grant from the Committee on Scientific Research of the American Medical Association.

tended to give slightly higher blood urea concentrations. We have designated these experiments as done under "special" conditions. The controls are given in Table I.

TABLE I.

The Ratio: $\frac{\textit{Urea in 1 Hour's Urine}}{\textit{Urea in 100 Cc. of Blood}}$ *in the Subject Dru. under Special Conditions Similar to the Standard Conditions except That No Urea Was Taken.*

Blood urea.	Urine urea.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.
mg. per 100 cc.	*mg. per hr.*	
20.3	972	47.9
21.1	1,020	48.4
22.0	1,040	47.3
24.0	1,080	44.8
26.1	1,140	43.7
26.1	1,190	45.6
26.3	1,177	44.8
27.0	1,210	44.8
27.6	1,200	43.5
28.0	1,425	50.9
29.1	1,320	45.3
30.0	1,480	49.3
32.1	1,560	48.6
Average		46.5
Standard deviation		±2.25
Variability		±4.95 per cent

The number of these observations is not large but they can nevertheless be regarded as a satisfactory control since the experimental changes we produced in the ratio pass well beyond the range of variation of the series. With these three average ratios and their variabilities as controls the effect of various factors on the rate of urea excretion was investigated.

1. Effect of Food.

In Table II the effect of a mixed meal on the ratio of subject Add. is shown.

The standard conditions were maintained while the first three measurements were being made. The new factor was introduced at 12.35 p.m. when lunch was taken. The effect is

not an immediate one, but the fifth ratio had risen to 60.9 and the sixth to 73.1. As the average ratio in Add. is 46.1 with a variability of ± 6.9 per cent, there can be no doubt but that the taking of food had in some way had the effect of increasing the

TABLE II.

Effect of a Mixed Meal.

Standard conditions (15 gm. of urea taken) until 12.35 p.m. when lunch was taken.

Subject: Add. Average control ratio = 46.1. Variability ±6.9 per cent.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg. per hr.	*mg. per 100 cc.*	*cc. per hr.*
10.35–11.29 a.m.	50.9	1,987	39.0	606
11.29–12.30 p.m.	49.6	1,710	34.5	606
12.30– 1.32 "	50.9	1,655	32.5	530
1.32– 2.50 "	48.1	1,478	30.8	378
2.50– 3.47 "	**60.9**	1,874	30.8	639
3.47– 4.42 "	**73.1**	2,193	30.0	822

TABLE III

Effect of a Mixed Meal.

Standard conditions (30 gm. of urea taken) until 12.30 p.m. when lunch was taken.

Subject: Dru. Average control ratio = 56.2. Variability ±4.8 per cent.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg. per hr.	*mg. per 100 cc.*	*cc. per hr.*
10.29–11.25 a.m.	61.2	3,492	57.0	489
11.25–12.25 p.m.	58.5	3,200	54.7	550
12.25– 1.25 "	54.6	2,820	51.7	304
1.25– 2.25 "	57.3	2,790	48.7	100
2.25– 3.28 "	65.3	3,037	46.5	105
3.28– 4.30 "	**71.4**	3,214	45.0	339
4.30– 5.19 "	**67.0**	3,012	45.0	559

rate of urea excretion. A similar experiment with the same result on Dru. is given in Table III.

Further experiments were carried out on Dru. in order to determine whether any particular food constituents were respon-

sible for the increase in the ratio observed after a mixed meal. These were carried out under special conditions and the control measurements are, therefore, those given in Table I in which the average ratio was 46.5 with a variability of ±4.95 per cent.

TABLE IV.

Effect of Cane-Sugar.

Special conditions (no urea taken) until 10 a.m. when 50 gm. of cane-sugar were taken.

Subject: Dru. Average control ratio = 46.5. Variability ± 5.0 per cent.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg. per hr.	*mg. per 100 cc.*	*cc. per hr.*
9.14–10.06 a.m.	49.5	1,120	22.6	635
10.06–11.10 "	48.0	1,028	21.4	324
11.10–12.10 p.m.	51.9	1,043	20.1	418
12.10– 1.01 "	51.3	970	18.9	547

TABLE V.

Effect of Whiskey.

Special conditions (no urea taken) until 10.25 a.m. when 100 cc. of whiskey were taken.

Subject: Dru. Average control ratio = 46.5. Variability ±5.0 per cent.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg. per hr.	*mg. per 100 cc.*	*cc. per hr.*
8.25– 9.25 a.m.	48.9	1,340	27.4	510
9.25–10.25 "	45.0	1,177	26.3	380
10.25–11.25 "	51.2	1,290	25.2	630
11.25–12.21 p.m.	47.2	1,136	24.1	630
12.21– 1.22 "	49.8	1,146	23.0	690

The ingestion of 50 gm. of cane-sugar did not have any appreciable effect on the ratio (Table IV). Another experiment with 100 gm. of "dextro-maltose" also gave negative results.

Whiskey taken in a single dose of 100 cc. had no very certain effect (Table V).

Coffee, on the other hand, had a definite effect (Table VI) and a similar result was obtained in another experiment in which 0.4 gm. of caffeine was taken.

Milk taken in large amounts was followed after a time by a pronounced increase in the ratio (Table VII).

Unfortunately, it was not possible to continue these experiments on the effect of various food constituents on the subject Dru. It was clear, however, that the effect of a mixed meal

TABLE VI.

Effect of Coffee.

Constant conditions (no urea taken) until 10 a.m. when 500 cc. of coffee were taken instead of water.

Subject: Dru. Average control ratio = 46.5. Variability ±5.0 per cent.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg. per hr.	*mg. per 100 cc.*	*cc. per hr.*
9.10–10.12 a.m.	49.8	1,150	23.1	532
10.12–11.12 "	54.5	1,200	22.0	275
11.12–12.14 p.m.	57.1	1,200	21.0	532
12.14– 1.14 "	56.7	1,133	20.0	600

TABLE VII.

Effect of Milk.

Constant conditions (no urea taken) until 9 a.m. when 500 cc. of milk were taken instead of water and again at 10 and 11 a.m.

Subject: Dru. Average control ratio = 46.5. Variability ±5.0 per cent.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg. per hr.	*mg. per 100 cc.*	*cc. per hr.*
8.30– 9.26 a.m.	46.5	1,270	27.3	509
9.26–10.26 "	48.7	1,340	27.5	495
10.26–11.24 "	49.8	1,380	27.7	481
11.24–12.20 p.m.	52.5	1,400	26.7	428
12.20– 1.16 "	63.6	1,430	22.5	477

was not due to the carbohydrate it contained, and on general grounds it seemed likely that the active constituent was protein. This supposition was confirmed by the following experiment on another subject which shows that a single amino-acid—glutamic acid—will increase the ratio in a manner which resembles the effect of mixed food (Table VIII).

TABLE VIII.

Effect of Glutamic Acid.

Standard conditions (20 gm. of urea taken) until 12.30 p.m. when 20 gm. of glutamic acid were taken.

Subject: B.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg. per hr.	*mg. per 100 cc.*	*cc. per hr.*
9.32–10.31 a.m.	52.1	2,600	49.9	534
10.31–11.30 "	57 8	2,660	46.0	778
11.30–12.29 p.m.	53.2	2,190	41.2	528
12.29– 1.30 "	60.4	2,330	38.6	521
1.30– 2.30 "	**66.4**	2,370	35.7	805
2.30– 3.26 "	**68.6**	2,420	35.3	794

2. Effect of Exercise.

Strenuous and prolonged exercise such as is involved in running continuously for 1 hour leads to a definite depression of the ratio. The decrease occurs only during the period in which the exercise is taken (Table IX).

TABLE IX.

Effect of Exercise.

Standard conditions (10 gm. of urea taken) until 9.37 a.m. when the subject began to run and continued the exercise without intermission until 10.35 a.m.

Subject: Dru. Average control ratio = 56.2. Variability ±4.8 per cent.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg. per hr.	*mg. per 100 cc.*	*cc. per hr.*
8.34– 9.36 a.m.	57.3	2,062	36.0	341
9.36–10.37 "	**42.3**	1,397	33.0	297
10.37–11.40 "	62.3	1,962	31.5	751
11.40–12.33 p.m.	61.5	1,845	30.0	394

3. Effect of Adrenalin.

In rabbits all the changes in the rate of urea excretion which cannot be explained by variation in blood urea concentration may be experimentally duplicated by the subcutaneous injection of adrenalin and of pituitrin. Those rates which are greater than the blood urea concentration would warrant can be pro-

duced by adrenalin (2) and those which are lower by pituitrin (3). Further, these substances, as far as their renal action is concerned, are directly antagonistic, for if the appropriate effective doses of adrenalin and pituitrin are mixed together before they are injected the rate of urea excretion is not changed (4).

In man it is not feasible to reproduce all of the adrenalin effects. The arterial system appears to be more sensitive to adrenalin in man than in the rabbit, at least very small subcutaneous injections have pronounced blood pressure and pulse rate effects in man, while it is reported that no amount has any effect on the blood pressure of rabbits when given subcutaneously (5). We found that a marked increase in the rate of urea excretion which could not be accounted for by any change in blood urea concentration was produced in rabbits by the subcutaneous injection 0.2 cc. of 1 in 1,000 adrenalin (Parke, Davis and Co.) per kilo body weight, while 0.1 cc. had no appreciable effect. These are much larger doses than can safely be given to human subjects. Such amounts as can be given without discomfort were used. In Add. and Dru. the subcutaneous injection of 0.01 to 0.015 cc. per kilo, as might have been expected, had no effect.

Adrenalin differs from pituitrin in that with increasing dosage there comes a point where the augmenting effect on the rate of urea excretion becomes less marked and as the dose is still further increased the effect is reversed and the rate is decreased. We have one experiment in man—in reality an accident—which illustrates this depressing effect of a very large dose of adrenalin. It was intended that the adrenalin should be injected under the skin of the subject's arm but by mistake 1 cc. of a 1 in 1,000 solution was injected into the cephalic vein. The intravenous injection of 1 cc. represents a much larger dose than any amount given subcutaneously, for there is evidence that the rate of absorption of adrenalin from the subcutaneous tissues is so slow that only a minute fraction of the injected adrenalin can at any one time be present in the blood stream. The subject became unconscious for a few minutes after the injection and when he recovered complained of a painful sense of constriction in his chest and of irregularity of the heart. During the hour following the injection there was a remarkable depression of the ratio (Table X).

TABLE X.

Effect of Adrenalin, Given Intravenously in Large Amounts.

Standard conditions (40 gm. of urea taken) until 10.02 a.m. when 1 cc. of 1 in 1,000 adrenalin (Parke, Davis and Co.) was injected intravenously. Subject: Az.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg per hr.	*mg. per 100 cc.*	*cc. per hr.*
8.00- 9.00 a.m.	56.6	3,270	57.8	443
9.00-10.00 "	51.7	2,790	54.0	500
10.00-11.00 "	**15.6**	820	52.5	28
11.00-12.00 n.	47.3	2,340	49.5	155
12.00- 1.00 p.m.	32.0	1,580	49.5	372

4. Effect of Pituitrin.

The results obtained in rabbits with pituitrin are easily reproduced in man because no unpleasant extrarenal effects are produced by the small amounts required to produce a marked depression of the rate of urea excretion. In Tables XI and XII the effect of the subcutaneous injection of 1 cc. of pituitrin (Parke, Davis and Co.) on the subjects Add. and Dru. is shown. The decrease in the ratio was not accompanied by any appreciable change in blood pressure or pulse rate.

TABLE XI.

Effect of Pituitrin.

Standard conditions (15 gm. of urea taken) until 10.35 a.m. when 1 cc. of pituitrin was injected subcutaneously. At 12.35 p.m. lunch was taken. Subject: Add. Average control ratio = 46.1. Variability ±6.9 per cent.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg. per hr.	*mg. per 100 cc.*	*cc. per hr.*
9.32-10.28 a.m.	48.0	2,015	42.0	617
10.28-11.27 "	**24.5**	1,027	42.0	208
11.27-12.27 p.m.	**21.7**	880	40.5	60
12.27- 1.40 "	**20.9**	847	40.5	35

TABLE XII.

Effect of Pituitrin.

Standard conditions (15 gm. of urea taken) until 11.05 a.m. when 1 cc. of pituitrin was injected subcutaneously.

Subject: Dru. Average control ratio = 56.2. Variability ±4.8 per cent.

Time.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea.	Blood urea.	Urine volume.
		mg. per hr.	*mg. per 100 cc.*	*cc. per hr.*
10.00-10.55 a.m.	60.8	2,695	44.3	652
10.55-11.55 "	**45.3**	1,890	41.7	130
11.55-12.45 p.m.	52.5	2,016	38.4	78

DISCUSSION.

The results of the experiments which have been given are an adequate explanation of the variability of the rate of urea excretion in the same individual under the ordinary conditions of every-day life, a variability which can only be partially accounted for by changes in the concentration of urea in the blood. They show that the ratio: $\frac{\text{urea in 1 hour's urine}}{\text{urea in 100 cc. of blood}}$ is increased by a mixed meal, by milk, by caffeine, and by glutamic acid, and that it is decreased by exercise and by large amounts of adrenalin. There are doubtless many other causes of variation so that under ordinary uncontrolled conditions we may conceive of the rate of urea excretion as constantly varying now above and now below the rate which is to be expected from the observed level of blood urea concentration as accelerating or inhibiting factors happen to predominate.

While the effect of the blood urea concentration is always apparent there are times when the effect of the other factors cannot be distinguished. These times are those in which certain special conditions are complied with, and the rate is then found to vary directly with the blood urea concentration alone. But it should be noted that this does not mean that these other factors are not at work, but simply that under certain conditions those factors which increase the rate are so counterbalanced by those which decrease it, that neither has any measurable effect. The most certain indication that both accelerating and inhibit-

ing factors are operative even when their influence is not apparent is the demonstration that a shift in the balance of the opposing factors occurs when the conditions are changed. With each alteration in the conditions the rate of urea excretion rises or falls, though at each new level of excretion a direct relation to the blood urea concentration is maintained so that it seems as though no other factor had any appreciable effect. These variations in the rate of urea excretion which cannot be accounted for by changes in blood urea concentration can only be maintained for any length of time under strictly controlled conditions. In the special experiments we have given and doubtless also under the ordinary conditions of every-day life they are very evanescent phenomena. A period of hyperactivity in urea excretion is followed shortly by a period of underactivity so that over any considerable length of time the effects of other factors than blood urea concentration tend to neutralize one another.

CONCLUSION.

The rate of urea excretion is increased by the administration by mouth of milk, caffeine, and glutamic acid, and is decreased by exercise, pituitrin, and large amounts of adrenalin. These alterations in the rate occur independently of changes in blood urea concentration.

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THE RATE OF UREA EXCRETION.

VIII. THE EFFECT OF CHANGES IN URINE VOLUME ON THE RATE OF UREA EXCRETION.*

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When an attempt was made some years ago in this laboratory to determine the effect of changes in urine volume on the rate of urea excretion no evidence of any definite relation between volume and rate was found (1). However, it was recognized that no very precise conclusions of any sort could be reached from the data then available, because the rates of urea excretion showed irregular variations under the influence of factors of an unknown nature (2). No adequate explanation for these fluctuations was found although it was clear that they were not due to changes in urine volume or in urine urea concentration. At a later date some of these unknown factors were defined by experimental work (3), but it was not until conditions had been found under which the effect of these disturbing factors was eliminated so that the ratio: $\frac{\text{rate of urea excretion}}{\text{concentration of urea in blood}}$ became approximately constant that the study of the influence of the volume of urine on the rate of urea excretion could be resumed with any hope of success. Now, however, when these conditions are complied with except for a variation in urine volume the effect on the rate will be measured by a deviation of the ratio from its constant value.

We have shown that the ratio: $\frac{\text{urea in 1 hour's urine}}{\text{urea in 100 cc. of blood}}$ remains nearly constant under conditions of which one of the require-

* This work was aided by a grant from the Committee on Scientific Research of the American Medical Association.

ments is the drinking of considerable amounts of water (4). The volumes of urine were large, but there was a considerable variation in the amount excreted per hour. In the subject Add. the range was from 155 to 684 cc. per hour and in Dru. from 220 to 840 cc. per hour. In neither of these subjects was the rate of urea excretion always exactly proportional to the blood urea concentration and the ratio was, therefore, not an absolute constant but had a variability of ±6.9 per cent in Add. and ±4.8 per cent in Dru. If this variability in the ratio is due to the variability of the urine volume, the relation should become evident when ratios are plotted against volumes. This has been done in Figs. 1 and 2.

The graphs show that the variations in urine volume had no appreciable effect on the ratio. This result confirms the conclusion reached by Austin, Stillman, and Van Slyke (5) that when urine volumes are large a variation in volume is without effect on the rate of urea excretion.

The question remains as to whether the rate may not be influenced when the urine volumes become small. A series of ratio measurements was therefore made on Dru. under conditions identical with those observed in the series detailed in Table I of the preceding paper with the exception that no water was given. The urine volumes were small and varied from 33 to 50 cc. per hour. The ratios, which are given in Table I, are of a lower value and are more variable than those obtained under other constant conditions, but the point of importance so far as the subject under discussion is concerned is that the variations in the ratio are in no way related to the variations in the urine volume, so that the only conclusion which can safely be drawn is the general one that the decrease in the ratio from 46.5, found when water was taken and the urine volumes were large, to 39.9 when no water was taken and the urine volumes were small, is in some way related to the abstention from water.

Another series of ratios from Dru. under different conditions, which were also associated with relatively small urine volumes, is given in Table II. In this case a 1 per cent sodium chloride solution was taken instead of water, but the conditions were in other respects the same as those under which the ratio averaged 46.5. In this case a change in the conditions consisting in the substitution of 1 per cent sodium chloride solution for water is

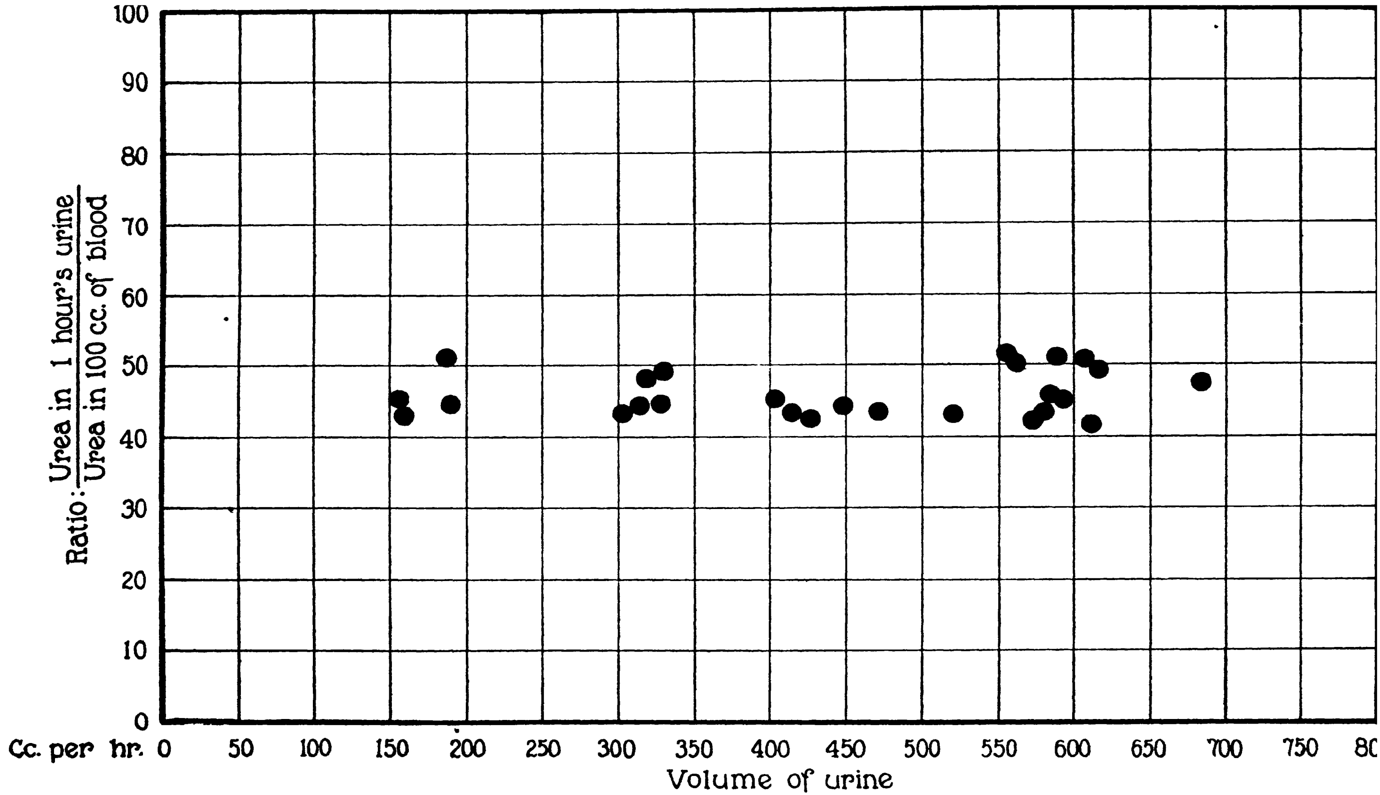

FIG. 1. Ratios on the subject Add. under standard conditions. There is no apparent relation between the ratio and the volume

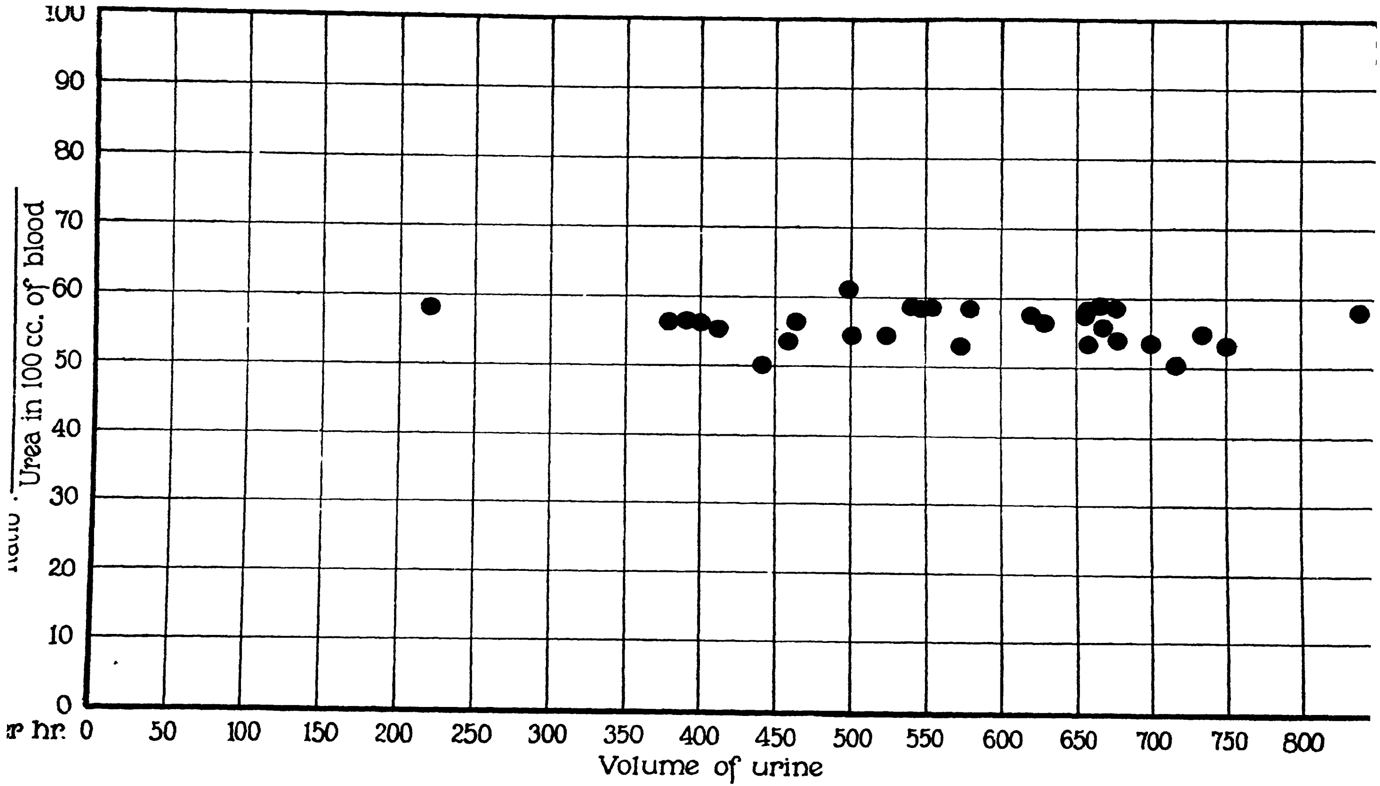

Fig. 2. Ratios on the subject Dru. under standard conditions. There is no apparent relation between the ratio and the volume.

TABLE I

The Ratio: $\dfrac{\textit{Urea in 1 Hour's Urine}}{\textit{Urea in 100 Cc of Blood}}$ *in the Subject Dru under Constant Conditions except That No Water Was Taken so That the Volumes of Urine per Hour Were Small.*

Volume of urine	Ratio $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea	Blood urea
cc per hr		*mg per hr*	*mg per 100 cc*
33	38.8	912	23.5
33	36.4	840	23.1
34	33.5	1,010	30.2
36	34.8	1,080	31.0
36	45.2	1,018	22.5
37	35.6	855	24.0
37	49.2	1,032	21.0
38	39.0	935	24.0
38	48.0	1,080	22.5
40	39.2	1,245	31.8
40	33.1	824	24.9
41	36.0	1,175	32.6
42	41.6	1,020	24.5
43	45.1	1,128	25.0
47	37.2	960	25.8
50	45.3	1,019	22.5

Average	39.9
Standard deviation	±5.1
Variability	±12.7 per cent

TABLE II

The Ratio: $\dfrac{\textit{Urea in 1 Hour's Urine}}{\textit{Urea in 100 Cc of Blood}}$ *in the Subject Dru under Constant Conditions except That Instead of Water a 1 Per Cent Solution of Sodium Chloride in Water Was Taken.*

Volume of urine	Ratio $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Urine urea	Blood urea
cc per hr		*mg per hr*	*mg per 100 cc*
64	55.7	1,338	24.0
82	61.5	1,390	22.6
83	54.3	1,222	22.5
89	53.0	1,272	24.0
110	60.6	1,280	21.1
127	62.4	1,410	22.6
Average	57.8		

TABLE III

The Ratio: $\frac{Urine\ Urea}{Blood\ Urea}$ *Measured When the Volume of Urine Was Small and the Concentration of Urea in the Urine Was High Compared with the Ratio:* $\frac{Urine\ Urea}{Blood\ Urea}$ *Measured When the Volume of Urine Was Large and the Concentration of Urea in the Urine Was Low.*

Subject.	Volume.	Concentration.	Small volumes and high concentrations of urea in urine. Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Large volumes and low concentrations of urea in urine. Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.
	cc. per hr.	*gm. per 100 cc.*		
McG.	35	4.38	24.1	45.4
A.	66	4.10	34.7	53.1
McB.	86	3.11	38.3	59.1
DeL.	65	3.21	28.8	43.4
H.	62	4.02	22.8	33.6
M.	62	3.87	29.2	47.6
L.	18	4.56	10.1	36.4
P.	53	3.96	19.2	25.6
R.	60	3.70	35.2	42.7
C.	51	4.09	35.0	48.3
S.	56	4.42	42.0	47.5
Co.	56	4.20	33.6	37.8
G.	86	1.23	12.3	12.0
L.	52	4.98	40.9	62.8
P.	43	1.96	12.5	16.0
W.	60	2.15	9.7	14.7
Col.	67	3.52	11.8	25.8
T.	43	4.46	36.8	61.5
Wa.	81	3.71	49.3	48.6
D.	81	3.54	52.8	48.0
Me.	49	3.89	23.9	31.3
Ca.	57	5.23	38.6	36.9
B.	42	2.62	32.3	56.5
Ga.	58	4.52	26.3	36.4
Ch.	41	4.23	20.0	37.5
I.	46	4.24	30.2	31.8
Gi.	40	2.45	7.1	15.1
H.	48	3.85	18.2	30.5
O'C.	39	6.18	25.6	42.6
He.	63	4.34	56.7	49.9
Su.	43	4.37	31.6	37.3

TABLE III—*Concluded.*

Subject.	Volume.	Concentration.	Small volumes and high concentrations of urea in urine.	Large volumes and low concentrations of urea in urine.
			Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.
	cc. per hr.	*gm. per 100 cc.*		
Wa.	70	3.42	34.0	37.5
Me	55	4.14	21.5	34.1
Hec.	42	4.12	25.7	40.6
Hel.	27	4.17	16.8	28.3
Mi.	58	4.14	23.6	54.3
Bo.	86	2.64	36.2	30.3
Gif.	73	2.74	30.4	46.9
Sc.	83	2.13	17.2	20.8
St.	47	4.83	32.2	38.6
Hes.	13	1.01	1.9	7.1
L.	56	3.02	23.8	45.5
F.	43	3.99	20.1	38.1
Br.	41	5.12	33.8	44.5
K.	20	3.99	25.0	34.9
Gi.	56	1.83	20.9	27.7
Ta.	27	3.84	23.2	48.3
Bur.	53	2.64	19.4	22.9
Gif.	35	2.10	11.9	33.8
An.	33	3.30	31.2	51.3
Average			26.8	38.0

associated with an increase in the ratio from 46.5 with large urine volumes to 57.8 with small urine volumes.

In the course of an unfinished investigation into the factors which influence the urea-concentrating capacity of the kidney we have collected further observations which have a bearing on the question of the effect on the rate of urea excretion of the reduction of the urine to small volumes. The ratio was measured under two sets of conditions after the administration of urea and water. Under one the measurement was preceded by a period of abstention from water and the urea was administered in only a small amount of water. Under the other set of conditions the urea was given in a large amount of water and additional water was taken while the ratios were being determined. In the one case the urine

volumes were very small and in the other case they were very large. Table III gives a comparison of the ratios under these two diverse conditions in normal individuals and in patients with Bright's disease. Table IV gives a similar comparison in normal rabbits.

TABLE IV.

The Ratio: $\frac{\textit{Urine Urea}}{\textit{Blood Urea}}$ *Measured When the Volume of Urine Was Small and the Concentration of Urea in the Urine Was High Compared with the Ratio:* $\frac{\textit{Urine Urea}}{\textit{Blood Urea}}$ *Measured When the Volume of Urine Was Large and the Concentration of Urea in the Urine Was Low.*

Rabbit No	Volume	Concentration	Small volumes and high concentrations of urea in urine. Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.	Large volumes and low concentrations of urea in urine. Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.
	cc. per hr.	*gm. per 100 cc.*		
1	3.4	4.87	1.78	2.26
2	4.5	3.72	2.38	3.13
3	2.9	5.84	1.94	2.24
4	5.9	5.64	3.13	3.09
5	3.2	4.87	2.83	2.49
6	3.2	5.03	3.72	2.94
7	2.9	6.66	3.37	2.72
8	3.5	7.08	3.98	3.07
9	5.2	3.03	1.73	2.41
10	5.3	6.86	2.93	2.73
11	3.7	6.35	2.24	2.48
12	2.0	7.61	2.53	3.34
13	2 2	7.31	3.50	2.42
14	3.5	6.87	3.35	2.54
15	2.3	5.00	3.67	2.53
16	2.7	4.79	3.07	3.45
17	1.5	4 06	1.33	2.62
18	1.9	6.09	2.66	2.48
Average			2.86	2.72

These tables show that in both human subjects and rabbits the variability of the ratio is greater when the conditions are such that the urine volumes are small. But as regards the magnitude of the ratio the results are diverse, for while the average ratio of

the human subjects is considerably reduced when the urine volumes are small, in rabbits the ratio is slightly though probably not significantly increased under the conditions associated with small volumes.

DISCUSSION.

We have given measurements of the rate of urea excretion on the same subject at known blood urea concentrations under four different but closely related conditions associated with wide variations in urine volume. In Table V the average ratios between the urine urea and the blood urea and the corresponding

TABLE V.

The Average Effect of Changes in Urine Volume on the Ratio: $\frac{\textit{Urea in 1 Hour's Urine}}{\textit{Urea in 100 Cc. of Blood}}$.

Observations on the subject Dru.

Conditions	No of observations	Average urine volume	Average ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$.
		cc. per hr.	
No water. No urea.	16	39	39.9
NaCl, 1 per cent. No urea.	6	92	57.8
Much water. No urea.	13	396	46.5
" " Urea.	31	555	56.2

average volumes are tabulated, and in Fig. 3 each ratio is separately charted against volume.

It is at once apparent that under no one of these conditions has the volume of urine any constant effect on the rate of urea excretion. The rate varies with the conditions under which the measurements were made, not with the volume.

On the other hand, the conclusion that the volume of urine is a factor, even an important factor, in determining the rate of urea excretion has been reached by other observers. Austin, Stillman, and Van Slyke (5) have given the most recent and the most specific definition of the effect of volume on rate in the statement that the rate of urea excretion increases in proportion to the square root of the urine volume per unit of time as long as the urine volume remains within ordinary limits. They measured the

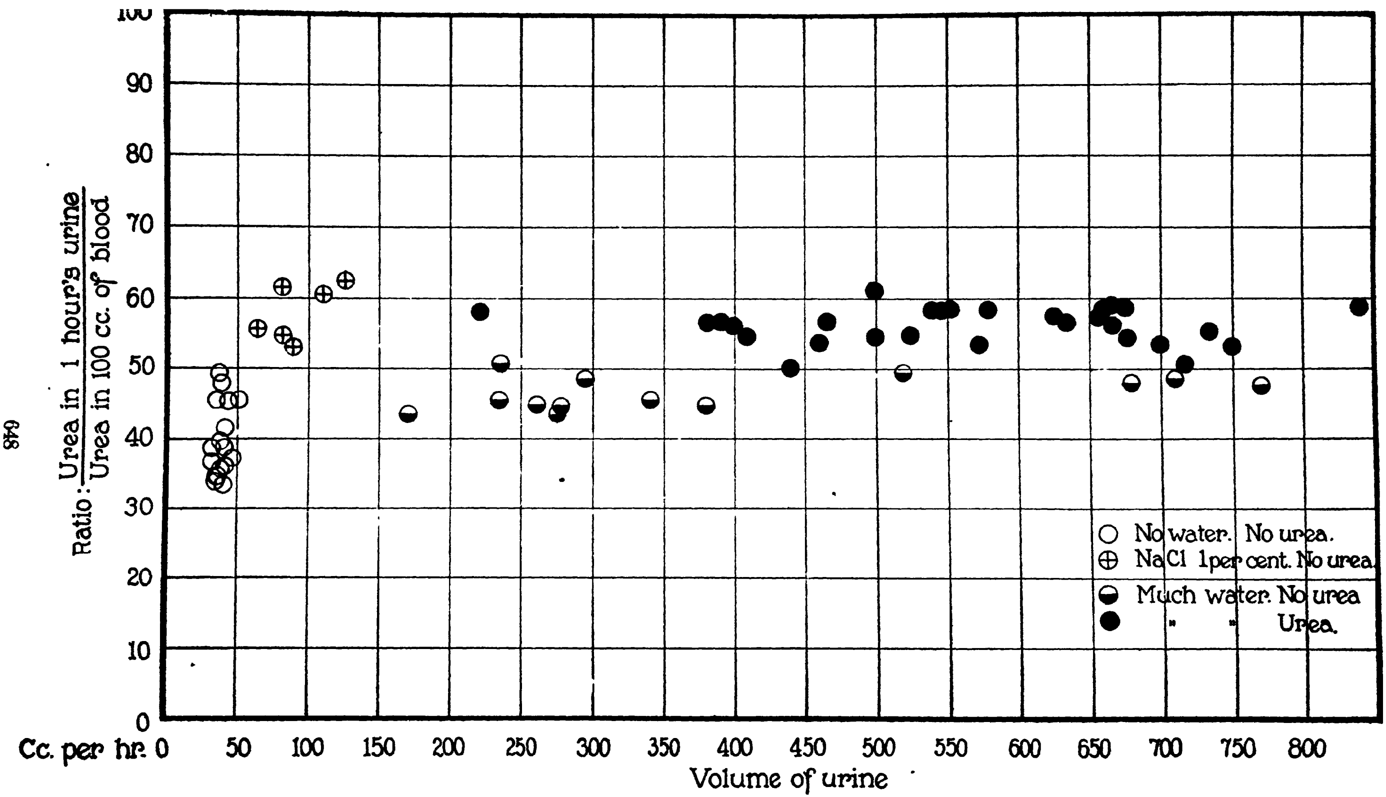

FIG. 3. Ratios on the subject Dru. under a variety of conditions. There is no apparent relation between the ratio and the volume.

rate of urea excretion in two normal subjects at varying blood urea concentrations and urine volumes, and studied the relation between the rate and the volume by plotting the ratio: $\frac{\text{urea in 24 hour's urine}}{\text{urea in 1,000 cc. of blood}}$ against the volume of urine per 24 hours. By utilizing additional data given by McLean (6) and by Addis and Watanabe (7) four graphs were constructed representing observations on four normal individuals. They found that in all these graphs the ratio tended to increase as the volume increased until a certain volume was reached which varied from 2,500 cc. per 24 hours in one subject to 6,000 cc. per 24 hours in another. They concluded that the rate varied with the square root of the volume because they found that their ratios became more constant if this particular mathematical expression were introduced as a factor. But the validity of this deduction is seriously prejudiced by the great variability of the data from which it was derived. As these authors themselves realized they were dealing with rates of urea excretion which were markedly influenced by other factors than either urine volume or blood urea concentration. This is sufficiently shown by the fact that the constant obtained from their formula $K = \frac{D}{B\sqrt{V}}$ (D being the rate of urea excretion, B the blood urea concentration, and V the volume) is found by them to vary in the same subject all the way from 27.4 to 75.1. But in any case it seems to us that the facts they record are more adequately explained in another way. We believe they have been misled through attributing to the volume effects which in reality were derived from certain conditions with which certain volumes are often, but not always, associated. Thus, they state that their small volumes of urine were obtained by avoiding food and water while their large volumes were secured by drinking water or 0.4 per cent salt solution. Somewhat similar conditions applied to that part of our previously published data which they utilized. In Paper VII (8) we have shown that the ratio is markedly increased by certain foods and here we have proved that the ratio is higher when water or salt solution is taken than when fluids are avoided, so that it was to be expected that they should find low ratios when neither food nor water was taken and a tendency to an increase in the ratio as the

conditions were altered and the activity of the kidney in the excretion of urea and of water increased. It is the frequent parallelism in the degree of activity of the kidney in the excretion of both urea and water which is confusing. But there is not necessarily any parallelism, and since we have failed to find that the volume has any effect on the rate when the conditions are kept constant, and have obtained unusually high rates of urea excretion with small urine volumes, there no longer seems to us any reason for supposing that the volume of urine is a factor in determining the rate of urea excretion.

We have shown that it is impossible to overtax the capacity of the kidney in the excretion of urea (9). It can also be shown that the limit of the renal capacity for water excretion cannot be reached. But there is a demonstrable limit to the urea-concentrating capacity of the kidney and it is this fact which makes the absence of any constant relation between the volume of urine and the rate of urea excretion an unexpected and remarkable finding. For in view of this limitation in concentrating capacity it might naturally be supposed that the rate would vary directly with the volume after the highest possible concentration of urea in the urine had been attained. The observations given in Tables III and IV were made in part in order to determine whether this expected relationship could be demonstrated. The subjects had been deprived of fluids and had been dehydrated by repeated administrations of urea until the concentration of urea in the urine had gradually risen to, or near to, a point beyond which no further reduction in urine volume or increase in urea excretion was effective in leading to still higher urine urea concentrations. But even under these extreme conditions there is no constant relation between the rate and the volume. In fact, in rabbits the ratio is slightly greater with maximal than with low urine urea concentrations. In the human subjects there is on the average a considerable reduction in the ratio when the urine urea concentrations are high, but in individual cases there is no semblance of any quantitative relation between the degree of decrease in the ratio and either the urine urea concentration or the volume. In some cases the decrease is marked, in others slight, and in 12 per cent of the observations there is an increase in the ratio when the urine urea concentration is high. The subject requires further

investigation, but these figures are given now in order to show that no definite relation between volume and rate can be demonstrated even when the conditions are such that on theoretical grounds a direct relationship might be expected.

CONCLUSION.

Changes in urine volume have no demonstrable effect on the rate of urea excretion.

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STUDIES ON ENZYME ACTION.

XXII. THE LIPOLYTIC ACTIONS OF VARIOUS TISSUE AND TUMOR EXTRACTS AT DIFFERENT HYDROGEN ION CONCENTRATIONS.

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INTRODUCTION.

The study of the enzyme actions of normal tissues and of malignant growths is being carried on because of the probable connection and possible dependence of growth upon such actions. The results which were obtained on protease actions were presented in two papers (1). For reasons which will be developed in a later paper, in place of continuing the study of the proteolytic enzymes of such materials, it was decided to investigate the lipolytic or ester-hydrolyzing enzymes. In this paper, the results which were obtained in attempts to determine the optimum conditions of hydrogen ion concentrations for the lipolytic actions of a number of tumor and normal tissue preparations on various esters will be presented.

The hydrogen ion concentration at which an enzyme shows its maximum action has been taken to be a characteristic property of that enzyme, provided certain conditions of testing are adhered to. While such optimum hydrogen ion concentrations have been determined for a number of enzyme preparations (2), no theory has as yet been proposed and generally accepted, which accounts for the existence of such optima on the basis of definite chemical or physical properties of the enzyme preparations used, or of the chemical reactions which are influenced by the enzymes. The change in activity of an enzyme with change in hydrogen ion

concentration must be looked upon, for the present at least, as an interesting and important characteristic even if of empirical nature.

The experimental determination of enzyme actions at different hydrogen ion concentrations and the fixing of the optimum conditions is comparatively simple for many enzymes. It is only necessary to add acid, alkali, or suitable buffer mixtures to bring about the desired condition, and to measure the activity for the necessary time periods. For such enzyme preparations as yeast sucrase, additions of foreign substances within reasonable limits, aside from change in hydrogen ion concentration, exert only small, if any, influence on the amount of action. At the other extreme are the lipases. Here, from the nature of the reaction influenced (hydrolysis of ester to form acid and alcohol), the hydrogen ion concentration of the mixture will be changed in most cases because of the acid produced in the reaction. Further, the acid, aside from its hydrogen ion concentration effect, may itself cause inactivation to a greater or less extent possibly because of negative ion or unionized molecule actions. The alcohol produced, as well as the original ester used, may also exert inactivating effects on the lipase. Different alcohols and different esters may have entirely different effects as shown in some studies on the castor bean lipase (3).

These relations indicate that the determination of the optimum conditions for the action of an enzyme as sensitive to added substances as lipase, and involving the formation of acid in the course of the determination, may lead to uncertain conclusions if the various possibilities are not kept in view.

Review of Previous Work.

The stalagmometric method of measuring lipolytic actions at different hydrogen ion concentrations was used by Davidsohn (4) and by Rona and Bien (5). Their determinations were made with buffer mixtures added to fix the hydrogen ion concentrations, mainly on triglycerides of a number of fatty acids at room temperatures for comparatively short time periods. The former investigator stated that with human duodenal contents, an optimum action was found at pH 8.5 and with pig pancreas extract at pH 8.0. A study of the experimental values and the curves represent-

ing these values does not indicate with certainty that a maximum value for the actions was reached, but that the trend of the results was still upward, and that, while the curves were clearly asymptotic, a limiting value had not been attained. The same relations hold for the results on blood serum and beef pancreas extracts, where optima were stated to exist at pH 8.4 and at pH 8.3 to 9.0, but with the same questionable foundation. Davidsohn found for human stomach contents acting on tributyrin an optimal zone at pH 4 to 5. While there is a real difference in the hydrogen ion concentration for optimum action for the latter lipase as compared with the former preparations, the experimental results given apparently do not permit of more than an approximate value for the stomach lipase.

Avery and Cullen (6) determined the conditions for optimum action on tributyrin of the lipase of pneumococcus dissolved in bile in the presence of suitable phosphate mixtures at 37° for 72 hours. The amount of acid formed was determined by titration. They stated: "The maximum activity of this esterase occurs at a reaction of about pH 7.8 and progressively decreases with increase in acidity." Results were not given for more alkaline solutions, so that this value cannot, without further evidence, be taken to be the optimum condition. They also found that exposure to acid reaction (pH 5.0) had little effect on the pneumococcus lipase after bringing it back to pH 7.0.

The results of Stevens and West (7) on the lipase of hemolytic streptococcus were obtained by methods similar to those used by Avery and Cullen. The optimum they gave for the actions, namely pH 7.9, is consequently also uncertain because more alkaline solutions were not studied.

Recently, Haley and Lyman (8) published some results indicating that castor bean lipase showed optimum action on olive oil at about pH 5.0. They appear to have limited their studies to the addition of acid to the castor bean preparation and did not study the possible effects of more alkaline conditions. The preparation without the addition of any acid had very little action. The hydrogen ion concentrations were determined on the filtrates, not on the mixtures as tested.

A review of the published results indicates that the knowledge of the optimum hydrogen ion concentrations for lipase actions is

in a very unsatisfactory state, partly because of the character of the chemical reaction which is involved. Indications of conditions for increased actions have been obtained, but beyond these nothing is definite.

EXPERIMENTAL METHODS AND RESULTS.

The influence of the hydrogen ion concentration on lipase was studied in two ways in the present investigation. In the first place, the extract was brought to different hydrogen ion concentrations with sodium hydroxide or hydrochloric acid, ester added, and the amount of hydrolysis measured by titration after a suitable time interval. In the second place, portions of the extract, after being brought to different hydrogen ion concentrations, were allowed to stand for from 12 to 24 hours, then all brought back to the same state, pH 7.0, ester was added, and the amount of hydrolysis measured after a suitable time interval.

The two methods do not determine the same property. The first method measures the amount of hydrolysis produced by the lipase material at different hydrogen ion concentrations, the blanks being determined for each hydrogen ion concentration. The second method measures the amounts of inactivation of the lipase material at different hydrogen ion concentrations, the activity tests and blanks all being determined at the same hydrogen ion concentration.

The hydrogen ion concentrations of the solutions were regulated by means of indicators and color comparison with standard solutions, the pH of which had been determined potentiometrically. Incubation of the lipase preparations with the esters was allowed to proceed from 21 to 24 hours at 37–38°.

The enzyme material was obtained from animal and human sources and included both tumor and normal tissues. A number of different tissues were investigated, not with the intention of making an exhaustive study of the optimum or favorable hydrogen ion concentrations for the lipolytic actions of all tissues, but in order to obtain a certain amount of representative data to indicate the possible significance of hydrogen ion concentration in these actions and to serve as a background for further studies of the tissue and other lipases.

Thirteen esters, the purest obtainable, were used, in the proportion of 3.4 milli-equivalents of the various esters in 15 cc. of mixture, made up as a rule, of 5 cc. of extract and 10 cc. of water plus hydrochloric acid or sodium hydroxide. After incubation, the amounts of hydrolysis or of acids produced, were determined by titration with 0.1 N sodium hydroxide solution with phenolphthalein as indicator. Suitable blanks were run in every case, and the amounts of the actions corrected for these. Toluene was present throughout the extractions and incubations.

Table I shows the various preparations used, their amounts, solid contents, and concentration of tumor extracted per cubic centimeter of final solution as incubated. The material was ground in a meat chopper or with sand in a mortar, extracted with water over night at 5–10°, centrifuged, and filtered through paper. The filtrates were then treated in the required ways and tested for lipase actions. The rat tumors were obtained from albino rats, both male and female, which were inoculated with the Flexner-Jobling rat carcinoma in the usual way (9) and allowed to grow for a period of from 3 to 6 weeks. The ages of the rats ranged from 50 to 150 days at the time of inoculation. The term $\frac{\text{FRC}}{\text{98A}}$ for example, indicates that the tumor used was derived from the 98th generation, Series A. The rats were fed on white bread soaked in whole milk, fresh cabbage or carrots, and tap water *ad libitum.* The neoplasm was removed from the animal after killing it with ether according to the method described in detail by Sugiura and Benedict (9). The natures of the tumors of human origin are given in Table I.

The solid contents were determined by heating small portions of the tissues or tumors in an air oven at 100–102°.

The experimental results will be presented in two ways: (1) As cubic centimeters of 0.1 N sodium hydroxide solution used in titrating the acid formed from the ester in the mixtures used; and (2) as relative actions calculated in terms of the action found at a definite hydrogen ion concentration.

In presenting the results, some detailed experimental values will first be given to show the magnitude of the various actions and in this way illustrate their probable accuracy. It will be impossible to list all of the results in this way because of lack of

space. The later tables will show all of the actions studied in relative and comparable form and show the various effects of the hydrogen ion concentrations.

The results given in Table II were obtained with rat cancer tissue extract and rat leg muscle extract on glyceryl triacetate at

TABLE I.

Data Relative to the Treatment of Materials for Lipase Experiments.

Experiment No.	Source of material.	Character of material.	Amount of material.	Solid contents of material.	H_2O used for extraction.	Tissue extracted per cc. of final solution.
			gm.	*per cent*	*cc.*	*mg.*
33	Rat.	$\frac{FRC}{98A}$	55.0	15.6	150*	49.0
41	Human.	Fibromyoma of uterus.	39.8	18.6	150†	82.0
49	Rat.	$\frac{FRC}{100E}$	31.2	15.7	100	39.3
50	"	Hind leg muscle tissue from Rat 49.	35.3	24.9	100	12.8
51	Human.	Benign villous papilloma of bladder.	6.8	17.3	75	20.8
52	Rat.	$\frac{FRC}{101A}$	26.3	16.5	150	74.0
53	"	Leg muscle tissue from Rat 52.	34.4	23.6	150	96.9
54	"	Leg muscle tissue of normal rats.	67.9	23.7	200	143 5
56	Human.	Fibromyoma of uterus.	185.8	16.1	400	159 0
M2	Beef.	Spleen.	360.0	26.0	1,000	87.0
R1	Human.	Splenomegaly fibrosis of spleen.	110.0	24.0	600	47.1
R4	Beef.	Suprarenal gland.	175.0	24.0	300	65.4
R5	Human.	Fibroids from uterus.	162.5	18.0	750	60.7
62	"	Fibromyoma of uterus.	259.0	16.1	400	100.3
81	Rat.	$\frac{FRC}{107C}$	38.0	15.0	200	32.3

* Dialyzed 19 hours after filtration.

† Dialyzed 6 hours after filtration.

different hydrogen ion concentrations and after different lengths of time; those given in Table III were obtained with a beef spleen extract and a human tumor extract, portions of which stood for 24 hours at low temperatures at the different hydrogen ion concentrations and then brought to pH 7.0 and tested on a number

of esters; and those given in Table IV were obtained with a human tumor extract tested on three esters in both ways.

The results in Table II show a steady increase in the relative amounts of actions as the solutions were made more alkaline

TABLE II.

Actions of Rat Tumor and Muscle Extracts on Glyceryl Triacetate at Different Hydrogen Ion Concentrations and for Different Time Periods.

	Experiment 49. Tumor extract.						
54 hrs. action.	Initial pH	4.0	5.0	6.0	7.0	8.0	9.0
	Final pH	4.0	5.0–5.2	5.0–5.2	5.0–5.2	5.0–5.2	5.0–5.2
	Action observed	0.16	0.35	0.73	0.74	0.92	0.99
	Relative action	**22**	**47**	**99**	**100**	**124**	**134**
24 hrs. action.	Initial pH	4.0	5.0	6.0	7.0	8.0	9.0
	Final pH	4.0	4.8–5.2	4.8–5.2	5.0–5.2	5.0–5.2	4.8–5.2
	Action observed	0.66	1.63	2.37	2.56	2.76	2.97
	Relative action	**26**	**64**	**93**	**100**	**108**	**116**
48 hrs. action.	Initial pH	4.0	5.0	6.0	7.0	8.0	9.0
	Final pH	4.0	5.3–5.5	5.3–5.5	5.3–5.5	5.3–5.5	5.3–5.5
	Action observed	1.14	2.69	3.71	3.96	4.10	4.51
	Relative action	**27**	**68**	**94**	**100**	**104**	**114**
	Experiment 50. Muscle extract.						
4 hrs. action.	Initial pH	4.0	5.0	6.0	7.0	8.0	9.0
	Final pH	4.0	5.0	5.0	5.6	5.8	6.4
	Action observed	0.02	0.08	0.16	0.22	0.20	0.43
	Relative action	**9**	**36**	**73**	**100**	**91**	**195**
23 hrs. action.	Initial pH	4.0	5.0	6.0	7.0	8.0	9.0
	Final pH	4.0	5.0–5.2	5.0–5.2	5.0–5.2	5.0–5.2	5.0–5.2
	Action observed	0.54	0.28	0.36	0.58	0.72	0.95
	Relative action	**93**	**48**	**62**	**100**	**124**	**164**
46 hrs. action.	Initial pH	4.0	5.0	6.0	7.0	8.0	9.0
	Final pH	4.0	5.0–5.3	5.3–5.6	5.3–5.6	5.3–5.6	5.3–5.6
	Action observed	0.88	0.42	0.56	0.68	1.02	1.27
	Relative action	**130**	**62**	**82**	**100**	**150**	**187**

initially; a change in hydrogen ion concentration during the actions in the solutions more alkaline than pH 5.0, all tending to become more acid up to about pH 5.0 to 5.5 where the buffer actions of the mixtures kept them at a constant value; and the same general

TABLE III.

Actions of Beef Spleen and Human Tumor Extracts at pH 7.0 on a Number of Esters after Standing at Different Hydrogen Ion Concentrations.

Experiment No. and material.	Ester.		pH at which mixtures were kept.					
			4.0	5.0	6.0	7.0	8.0	9.0
M2	Phenyl acetate.	Action observed.	2.11	3.16	6.98	5.90	4.46	2.76
Beef spleen.		Relative action.	**30**	**45**	**100**	**85**	**64**	**40**
	Glyceryl triacetate.	Action observed	2.39	3.01	4.34	4.32	3.71	2.96
		Relative action.	**55**	**70**	**100**	**100**	**85**	**68**
	Methyl butyrate.	Action observed.	0.09	0.33	0.58	0.98	0.41	0.16
		Relative action.	**9**	**34**	**60**	**100**	**42**	**16**
	Benzyl acetate.	Action observed.	0.25	0.44	0.85	0.95	0.83	0.76
		Relative action.	**26**	**46**	**90**	**100**	**87**	**80**
	Ethyl acetate.	Action observed.	0.30	0.39	0.62	0.70	0.58	0.50
		Relative action.	**43**	**56**	**89**	**100**	**83**	**71**
	Methyl acetate.	Action observed.	0.42	0.49	0.87	0.80	0.70	0.56
		Relative action.	**48**	**56**	**100**	**92**	**80**	**64**
	Ethyl butyrate.	Action observed.	0.08	0.29	0.63	0.69	0.23	0.10
		Relative action.	**12**	**42**	**91**	**100**	**33**	**14**
	Methyl benzoate.	Action observed.	0.04	0.14	0.12	0.09	0.05	0.07
	Ethyl benzoate.	" "	0.04	0.10	0.16	0.03	0.02	0.04
	Isobutyl acetate.	" "	0.00	0.03	0.28	0.24	0.10	0.00
		Relative action.	**0**	**11**	**100**	**86**	**36**	**0**
	Triphenyl phosphate.	Action observed.	0.00	0.07	0.06	0.00	0.00	0.00
	Olive oil.	" "	0.03	0.04	0.03	0.02	0.00	0.00
	Methyl salicylate.	" "	0.13	0.21	0.15	0.10	0.08	0.07
62	Phenyl acetate.	" "		1.08	1.25	1.24	1.20	1.05
Human tumor.		Relative action.		**86**	**100**	**99**	**96**	**84**
	Glyceryl triacetate.	Action observed.		0.73	0.85	0.82	0.83	0.64
		Relative action.		**86**	**100**	**96**	**98**	**75**
	Methyl butyrate.	Action observed.		0.27	0.43	0.44	0.41	0.35
		Relative action.		**61**	**98**	**100**	**93**	**80**
	Benzyl acetate.	Action observed.		0.18	0.23	0.24	0.26	0.20
		Relative action.		**70**	**88**	**92**	**100**	**77**
	Ethyl acetate.	Action observed.		0.14	0.21	0.26	0.20	0.16
		Relative action.		**54**	**81**	**100**	**77**	**62**
	Methyl acetate.	Action observed.		0.23	0.30	0.36	0.32	0.23
		Relative action.		**64**	**83**	**100**	**89**	**64**
	Ethyl butyrate.	Action observed.		0.00	0.06	0.04	0.06	0.00

TABLE III—*Concluded.*

Experiment No and material.	Ester.		pH at which mixtures were kept.					
			4 0	5 0	6.0	7 0	8 0	9 0
	Methyl benzoate.	Action observed.		0 10	0 17	0 17	0 15	0 15
	Ethyl benzoate.	" "		0 04	0 07	0 06	0.00	0 00
	Isobutyl acetate.	" "		0 00	0 00	0 00	0 00	0 00
	Triphenyl phosphate.	" "		0 00	0 00	0 00	0 00	0 00
	Olive oil.	" "		0 00	0 05	0 02	0 06	0 02

behavior for the actions at different time intervals, the relations becoming more marked and clearer for the longer periods. The actions for the tumor extracts were greater than for the muscle extracts and the relations therefore clearer because of the relatively smaller experimental errors, but similar results were obtained with both. The relation between amounts of action and times will be spoken of in a subsequent paper. The general conclusion to be drawn from the fact of starting the actions at different hydrogen ion concentrations which, in the course of the actions tended to reach the same value, is that the inactivations at the different hydrogen ion concentrations occurred at different rates. For purposes of comparison, the values found at pH 7.0 were placed equal to 100, and the other results calculated in terms of these.

The small increases in alkalinity observed with some of the mixtures in the time periods between 24 and 48 hours may have been due to autolytic changes of the material, liberating small amounts of ammonia or possibly basic amino-acids or split protein products. Such decompositions undoubtedly would also occur in the blanks, and the lipolytic actions as given would be corrected for such possible changes.

The results given in Table III in which the preparations were kept at different hydrogen ion concentrations and then all brought to pH 7.0 and tested on a number of different esters show, in every case in which appreciable action was found, that an optimum condition existed. Kept at more acid or more alkaline conditions than this optimum, inactivation resulted,

TABLE IV.

Comparative Actions of Human Tumor Extract (Experiment R5) on Esters at Different Hydrogen Ion Concentrations and at the Same Hydrogen Ion Concentration after Standing at Different Hydrogen Ion Concentrations.

Ester.		Direct tests at indicated pH.						Standing at indicated pH and tested at pH 7.0.					
		4.0	5.0	6.0	7.0	8.0	9.0	4.0	5.0	6.0	7.0	8.0	9.0
Phenyl	Action observed.	0.19	0.76	0.81	1.09	1.14	1.42	0.22	0.88	1.09	1.11	1.10	0.95
acetate.	Relative action.	**17**	**71**	**74**	**100**	**105**	**130**	**20**	**79**	**98**	**100**	**99**	**86**
Glyceryl	Action observed.	0.87	0.24	0.53	0.89	0.89	1.20	0.24	0.54	0.78	0.77	0.66	0.55
triacetate.	Relative action.	**98**	**27**	**60**	**100**	**100**	**135**	**31**	**70**	**100**	**99**	**85**	**71**
Methyl	Action observed.	0.35	0.18	0.47	0.63	0.57	0.74	0.12	0.59	0.78	0.79	0.78	0.65
butyrate.	Relative action.	**56**	**29**	**75**	**100**	**90**	**117**	**15**	**75**	**99**	**100**	**99**	**82**

greatest in the most acid and alkaline solutions. The amounts of sodium chloride formed in the treatments in these and similar experiments were too small to influence the actions appreciably. The optimum zones were more or less extended to judge from the results, and the optima, themselves, were not far removed from pH 7.0 in most of the tests, although a number were found to be at pH 6.0.

TABLE V.

Comparative Actions of Different Preparations on Esters at Different Hydrogen Ion Concentrations.

Experiment No.	Source.	Ester.	pH					
			4.0	5.0	6.0	7.0	8.0	9.0
33	Rat tumor.	Glyceryl triacetate.	64	75	95	100	99	104
49	" "	" "	26	64	93	100	108	116
52	" "	" "		46		100		
41	Human "	" "	39	45	57	100	123	120
51	" "	" "		53		100		
R5	" "	" "	(97)	27	59	100	100	135
56	" "	" "		27		100		
50	Rat muscle.	" "	(94)	48	62	100	124	163
53	" "	" "		27		100		
54	" "	" "		47		100		
33	" tumor.	Ethyl butyrate.	(81)	0	41	100	124	146
56	Human "	" "		0		100		
56	" "	Methyl "		14		100		
56	" "	" acetate.		16		100		
56	" "	Ethyl "		21		100		
56	" "	Benzyl "		60		100		
56	" "	Phenyl "		37		100		
56	" "	Methyl benzoate.		92		100		

The results in Table IV on the same extract by the two methods show the same relations as the results in Tables II and III; greater actions with increased alkalinity when tested at the different hydrogen ion concentrations; definite optima in the neighborhood of pH 7.0 when allowed to stand at different hydrogen ion concentrations and then tested at pH 7.0.

The results so far presented show the magnitude and nature of the actual experimental results obtained. The actions as shown

give the actual cubic centimeters of 0.1 N alkali required to neutralize the acid formed, suitably corrected for blanks. In giving the results of the remaining experiments on this subject, the detailed experimental data will not be given. The comparative actions for the different hydrogen ion concentrations will be listed since the actual values found are similar to those already given.

In Table V are shown all the results obtained on different esters with the mixtures brought to the indicated hydrogen ion concentrations and tested. The action at pH 7.0 in any one series was arbitrarily placed at 100 and the others in that series calculated in terms of this.

These results show no optimum hydrogen ion concentration for the actions on glyceryl triacetate and on ethyl butyrate (one experiment), the other esters not having been tested in solutions more alkaline than pH 7.0. In the more acid solutions, three preparations showed a greater activity at pH 4.0 than at pH 5.0. Whether an optimum exists in more acid solution cannot be stated on the basis of these results, but it seems to be true that for some preparations there is greater action at pH 4.0 than at pH 5.0. It may also be noted that, starting from pH 7.0, the actions on the butyrate decreased more rapidly in the more acid solutions than the actions on glyceryl triacetate.

In Table VI are shown the comparative actions where the various preparations were tested at pH 7.0, after having been allowed to stand at the different hydrogen ion concentrations as indicated, the largest action in any one series being placed equal to 100.

The results in Table VI show definite optima for the different esters and preparations, either pH 6.0 or 7.0 in every case. A number of preparations from different sources were used, but this general relation was found in every case.

A closer study of the results reveals several further regularities. Because of the possible experimental errors which are magnified in the smaller actions, a certain amount of irregularity in the results in the more acid and alkaline solutions is to be expected. At the same time, an attempt to average the results for any one hydrogen ion concentration and one ester might involve values so different in magnitude that the method might well be open to

TABLE VI.

Comparative Actions of Preparations at pH 7.0 after Standing at Different Hydrogen Ion Concentrations.

Experiment No.	Source.	Ester.	pH at which mixtures were kept.					
			4.0	5.0	6.0	7.0	8.0	9.0
62	Human tumor.	Glyceryl triacetate.		88	100	96	98	77
R5	" "	" "	30	68	100	99	84	70
81	Rat "	" "	82	91	96	100	93	90
R1	Human spleen.	" "	61	83	100	91	75	76
M2	Beef "	" "	55	69	100	99	85	68
62	Human tumor.	Phenyl acetate.		87	100	99	96	84
R5	" "	" "	20	80	98	100	99	85
81	Rat "	" "	83	93	94	100	91	88
R1	Human spleen.	" "	64	90	100	95	83	80
M2	Beef "	" "	30	45	100	84	64	40
R4	Suprarenal gland.	" "	87	89	100	78	54	56
62	Human tumor.	Benzyl "		71	88	94	100	76
R1	" spleen.	" "	41		96	100	92	41
81	Rat tumor.	" "	41	100	100	94	97	93
M2	Beef spleen.	" "	26	47	90	100	87	70
R4	Suprarenal gland.	" "	82	96	100	96	84	101
62	Human tumor.	Methyl "		63	83	100	88	63
R1	" spleen.	" "	45	68	100	98	73	68
M2	Beef "	" "	48	57	100	91	81	64
R4	Suprarenal gland.	" "	72	100	91	77	67	67
62	Human tumor.	Ethyl "		53	83	100	88	63
R1	" spleen.	" "	38	61	94	100	80	72
M2	Beef "	" "	43	54	87	100	81	70
R4	Suprarenal gland.	" "	65	100	98	93	75	63
62	Human tumor.	Methyl butyrate.		62	100	100	93	79
R5	" "	" "	15	75	99	100	99	82
81	Rat "	" "	22	78	100	95	83	80
R1	Human spleen.	" "	8	68	72	100	78	30
M2	Beef "	" "	9	33	59	100	41	16
R4	Suprarenal gland.	" "	33	69	100	65	48	17
R1	Human spleen.	Ethyl "	8	62	68	100	61	40
M2	Beef "	" "	11	42	91	100	34	15
62	Human tumor.	Methyl benzoate.		64	100	100	91	91
R1	" spleen.	" "	0	47	91	100	74	47
R1	" "	Ethyl "	16	54	92	100	81	55

criticism. Some conclusions will therefore be suggested, based on the results as given, with the knowledge that the irregularities in the data permit of no more exact relations at present.

In comparing the acetic esters with the butyric esters, it is evident that in the more acid solutions, the activity loss is much greater with the latter than with the former. The benzoic esters may be grouped with the butyric esters. The optimum pH values for the various actions are not sharp, but represent zones or ranges rather than points if curves were plotted. Within the various groups of esters only a few regularities are observable. Thus, with methyl butyrate on the alkaline side, the three tumor extracts showed only small losses in activity while the three non-tumor extracts showed much greater losses for the same ranges.

DISCUSSION OF RESULTS.

Two definite conclusions may be drawn from the experimental results presented in this paper. (1) The lipolytic actions of the tissue and tumor extracts show no optimum at any hydrogen ion concentration tested, but increase in magnitude as the alkalinity of the mixtures increases. (2) The rates of inactivation of the various mixtures are least in the neighborhood of pH 6.0 to 7.0 and increase in more acid and more alkaline solutions.

In testing the lipolytic activity of the extracts described here, two factors are operating simultaneously in every case; enzyme action at a definite hydrogen ion concentration, and inactivation of enzyme at that hydrogen ion concentration. To complicate the interpretation of the results, in all mixtures except those initially at pH 4.0 and 5.0, the hydrogen ion concentration was changing as the reactions proceeded, mainly because of the acids formed in the hydrolysis of the esters. These changes in the mixtures studied continued until the pH values reached 5.0 to 5.5, when the buffer actions appeared to be sufficient to prevent further changes.

The difficulty of an exact interpretation of the most favorable hydrogen ion concentration for such lipase actions is therefore apparent. The study of the actions at different hydrogen ion concentrations, definite at the commencement of the actions, involves changes in the reactions and therefore gives results under

changed, or continuously changing, conditions. The study of the rates of inactivation by testing at the same hydrogen ion concentration, after permitting the mixtures to stand at different hydrogen ion concentrations, yields definite conclusions, but really involves a problem essentially different from that of actions at definite hydrogen ion concentrations.

These relations hold also for the results of others. The obvious way out of the dilemma is to carry out the studies in the presence of buffer mixtures which will allow of only small changes in hydrogen ion concentrations. Leaving aside the question of the experimental difficulties involved in such a method with lipase, the fact that many of the lipase actions which have been studied in the past have been found to be sensitive to the presence of added inorganic salts, makes the presence of buffer substances a complicating factor in place of simplifying the conditions.

It is, of course, possible that the comparative rates of action and of inactivation of certain lipase preparations may be such that definite optima are obtained at certain hydrogen ion concentrations. In the experiments described here, it is seen that the greater actions in the more alkaline solutions more than make up for the inactivations in those solutions, while in the more acid solutions, the reverse is the case. Similar reasoning may be applied to the results of others described in the earlier part of this paper.

Aside from these general considerations, little need be added with reference to the discussion of the separate experiments and preparations described in the experimental part. No essential differences were observed in the behavior of the extracts of the various tissues and tumors with regard to the influence of the hydrogen ion concentration, and while only a limited number were tested, the results were so similar, that, until further evidence is available, the relations may be considered to hold for similar preparations. The fact that some minor differences were observed with different esters, such as more rapid inactivation of the preparations toward the butyrates and benzoates than toward the acetates in the more acid solutions, and the less rapid inactivation of the tumor extracts than some of the tissue extracts toward methyl butyrate in the more alkaline solutions, is of interest but does not invalidate the general conclusions.

The writers wish to thank Dr. A. B. Reese of The Roosevelt Hospital Staff and the Members of the Surgical Staff of Memorial Hospital for their courtesy in furnishing materials from surgical operations for certain of the experiments. Thanks are also due Mr. Isaac Lorberblatt for assistance in carrying out the experiments.

SUMMARY.

The uncertainty of the results of the determinations of the optimum hydrogen ion concentrations of lipase actions recorded in the literature is pointed out.

Lipase actions of various tumor and tissue preparations on a number of esters were determined at different hydrogen ion concentrations and at the same hydrogen ion concentration after having been kept at different hydrogen ion concentrations. The various materials and preparations used gave similar results.

No optimum conditions were found for the actions at different hydrogen ion concentrations, the amounts of the actions increasing continuously as the solutions became more alkaline, up to pH 9.0.

Definite optima, in the neighborhood of pH 6.0 to 7.0, were found for all the preparations when tested under the same conditions at pH 7.0, after having stood at different hydrogen ion concentrations.

Where appreciable action was shown, the optima with the different esters were the same.

Minor differences in the actions with certain of the esters were pointed out.

The relation between lipolytic actions at different hydrogen ion concentrations and rates of inactivation of lipase at different hydrogen ion concentrations was discussed briefly, as well as the difficulty of obtaining satisfactory results for the hydrogen ion concentration for optimum lipase action.

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SOME DERIVATIVES OF CYSTINE AND CYSTEINE.

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Several months ago we undertook, from an anabolic as well as from a catabolic standpoint, a study of the metabolism of cystine. Under the former aspect, we wished to consider the possibilities of the synthesis of cystine in the animal body, when, namely, under conditions of bromobenzene poisoning, various forms of nitrogen and sulfur were supplied. Viewed catabolically, we desired to study the oxidation of cystine as accomplished by the normal organism.

In connection with this work we prepared a number of derivatives of cystine and cysteine, which we thought might be of interest to other workers in this field. Accordingly, we have noted briefly the names of the various compounds, their methods of preparation, and some of the more important of their physical constants, together with sufficient analytical data in each case to identify the substance in question. As this preparation work, however, was entirely of a secondary nature, no attempt was made to make an exhaustive study of the compounds.

1. Cystine.

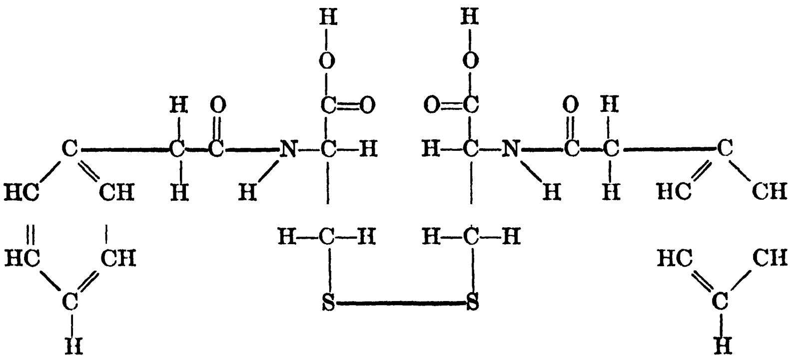
In the preparation of this material we followed in general the method outlined by Folin (1), and modified by Lewis (2), to which we added one or another of our own alterations. Ordinary barber shop sweepings made a sufficiently satisfactory starting material. The hair (without washing), was digested on the water bath in a 6 liter Pyrex Florence flask with concentrated hydrochloric acid (100 cc. of acid for each 100 gm. of hair) until apparent solution was effected (about 2 hours). Thereupon, the mixture was refluxed over a free flame for about 8 hours longer, *i.e.* until hydrolysis was completed, as evidenced by a

negative biuret. After cooling, the contents of the flask were filtered through glass wool, transferred to a large evaporating dish, and *almost neutralized* with saturated commercial sodium hydroxide solution; *i.e.*, until Congo red turned purple instead of blue. Solid technical sodium acetate was then added until the Congo red test was negative for mineral acids. The material was then allowed to stand over night, or longer, before filtering. We found, in every case, that about 12 hours were sufficient for the cystine to precipitate, and under these conditions we never had any tyrosine to bother us in the final purification. After filtration, the residue was transferred to a large Pyrex beaker, covered with about 500 cc. or more of 5 per cent hydrochloric acid, heated to boiling, and decolorized with animal charcoal until the filtrate was water-clear. This filtrate was then treated the same as the very first filtrate; *i.e.*, neutralization was *begun* with technical sodium hydroxide solution and completed with *hot* saturated sodium acetate solution. Under no conditions should the hot cystine solution ever be made alkaline with sodium hydroxide solution. A very bulky precipitate, which was mostly inorganic matter, formed on this last neutralization. After cooling, this was filtered, the residue transferred to a large beaker and stirred up well with diluted ammonium hydroxide (1 part in 5). This dissolves the cystine, but does not remove any of the inorganic matter, especially not the calcium phosphate. Finally, the ammoniacal solution was filtered, and the cystine precipitated by neutralization with acetic acid. The substance was recognized by its characteristic hexagonal crystals. It was a very pure product as evidenced by the Kjeldahl nitrogen determinations and by the microscopical crystal appearance. We averaged about 6 to 6.5 gm. of cystine per 100 gm. of hair.

It not infrequently happens that a large amount of the cystine is retained by the charcoal when the decolorizing mixture is filtered. In such a case this residue should be boiled again with diluted hydrochloric acid, filtered, and the filtrate worked up as above. One may easily ascertain the presence or absence of cystine in the charcoal residue by fusing a small amount (2 gm.) of the mass in a crucible with a half inch stick of potassium hydroxide. After heating it for a few minutes, wash it into a

test-tube, and acidify it strongly with sulfuric acid. If the odor of hydrogen sulfide is imperceptible or only faint, there was little or no cystine present.

2. Diphenylacetyl Cystine.



This substance was prepared according to the Schotten and Baumann synthesis as follows:

The cystine was dissolved in 10 per cent sodium hydroxide solution, diluted with about an equal volume of water, and put into a suitable bottle which could be corked tightly and easily shaken. Phenylacetyl chloride was then added in small amounts (0.5 cc.) until a total of about $1\frac{1}{2}$ molecules was used for each molecule or cystine. The container was vigorously shaken after each addition of the acid chloride, and the solution was kept slightly alkaline by the addition of sufficient saturated sodium hydroxide solution at each addition of the acid chloride. The reaction proceeded quite rapidly, and at times it was necessary to cool the bottle under the tap. When the operation was completed, the contents of the bottle were rinsed into a separatory funnel and acidified to Congo red with concentrated hydrochloric acid. Immediately a heavy, gummy, white mass precipitated. The mother liquor was then drained off, the precipitate dissolved in diluted alcohol (40 per cent) and filtered. Any uncombined ncystine was thus removed. The filtrate was then heated to boiling and very carefully diluted with *hot* water until a permanent milkiness appeared. Thereupon, the container was placed in a larger vessel of hot water to insure a very gradual cooling

and consequently a better crystallization. The precipitate which formed was filtered by suction, boiled with carbon tetrachloride to remove the free phenylacetic acid which had formed during the reaction, refiltered, washed with fresh carbon tetrachloride, and finally recrystallized from hot water, from which it separates as pure white, fine, short rods or needles. After drying *in vacuo*, the substance melted sharply at 119–121°C.

The compound was soluble in *hot* water to the extent of about 7 parts in 1,000, but was just about insoluble in cold water. It was exceedingly soluble in both alcohol and acetone. It was quite insoluble in cold benzene and only slightly soluble in the hot solvent. In petroleum ether, however, carbon tetrachloride, and ether it was almost insoluble, both cold and hot. The yield was about 80 per cent.

Dried *in vacuo* at 75–80°, the substance gave the following results by the Kjeldahl method.

Calculated.	N 5.88,	S 13.44.
Found.	" 6.02, 6.08;	" 13.60, 13.74.

3. Cystine Phenylhydantoic Acid (Phenyluramino Cystine).

In this synthesis the directions given by Patten (3) were followed. The operation is very simple and the results are almost quantitative. The final product, obtained and purified by diluting its acetone solution with water, was the same as that originally prepared by Patten (3). It crystallized in beautiful, fine, long, feathery crystals (M.P. 160°C.).

It was very soluble in alcohol, acetone, and alkalies, and moderately soluble in both cold and hot glacial acetic acid. On greatly diluting the glacial acetic solution with water, the dissolved substance slowly precipitated in crystalline form. The compound was very insoluble, however, in the following solvents, both cold and hot: benzene, ethyl acetate, carbon tetrachloride, ether, water, and mineral acids.

4. Cystine Phenylhydantoin.

The transformation of the phenyluramino cystine into cystine phenylhydantoin, by a dehydration of the former, is very simple

and easy (3). The phenyluramino cystine was warmed in 10 per cent hydrochloric acid on the water bath for a couple of hours. After cooling, the mixture was filtered and the residue dissolved in a small amount of *hot* 95 per cent alcohol. As the hydantoin is very insoluble in *cold* alcohol, the filtration had to be carried out *very rapidly*. Upon cooling, the substance crystallized very quickly, in the form of fine, short needles, it was recrystallized a couple of times from alcohol. A very beautiful, white product was thus obtained, which melted, when dried *in vacuo*, sharply at 117°C.

The substance was insoluble in cold benzene, but slightly soluble in the hot solvent. It was likewise insoluble in cold ethyl acetate, but dissolved easily on heating. In acetone it was very soluble in the cold, and recrystallized on diluting the solution with water. In glacial acetic acid it was insoluble in the cold, but dissolved quite readily on warming, and recrystallized on cooling. In carbon tetrachloride, ether, water, and alkalies it was insoluble both cold and hot.

Dried *in vacuo*, the substance gave the following results for nitrogen by the Kjeldahl method.

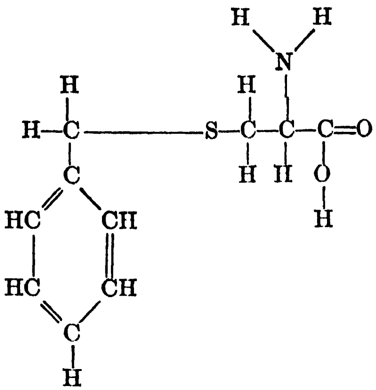
Calculated.	N 12.90.
Found.	" 13.01, 13.09.

5. Cysteine.

The substance was obtained according to the usual method, namely by reducing cystine with tin and concentrated hydrochloric acid, removing the tin with hydrogen sulfide, and evaporating to dryness on the water bath. The material thus obtained was insoluble in the following organic solvents, both hot and cold: ether, acetone, ethyl acetate, benzene, carbon disulfide, and carbon tetrachloride. It was very soluble in water and alcohol, especially on warming. According to the Kjeldahl method it contained the proper percentage of nitrogen.

Due to the facility with which cysteine oxidizes, in neutral and alkaline solution, to cystine, we employed the additive hydrochloride compound whenever possible, which we kept in stock as a water solution.

6. Benzyl Cysteine.



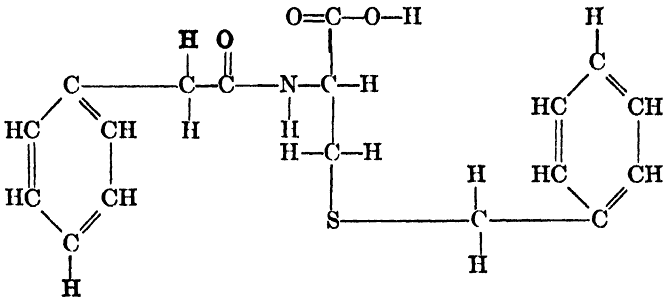
The synthesis was effected according to the simple method described by Suter (4). The water solution of the cysteine hydrochloride was placed in a suitable bottle and treated with a bit more than the theoretical amount of benzyl chloride. After the addition of an excess of sodium hydroxide (somewhat more than 3 molecules for each molecule of cysteine), the container was shaken vigorously for about half an hour. The material was then transferred to a separatory funnel and extracted several times with ether to remove the excess benzyl chloride. Upon acidifying weakly with acetic acid the benzyl cysteine crystallized out at once. It was then filtered and recrystallized from hot water, from which it separated as white, pearly leaflets, which melted at 215°C., with browning and decomposition.

The compound was easily soluble in mineral acids and in alkalies, as also in hot water, but not in cold water. It dissolved readily in glacial acetic acid, especially on warming, and did not precipitate on cooling or slightly diluting with water, but on great dilution with water it precipitated slowly. It was very insoluble in ether, alcohol, ethyl acetate, petroleum ether, acetone, carbon tetrachloride, carbon disulfide, and benzene.

Dried *in vacuo* at 90°, the substance gave the following analytical results by the Kjeldahl method.

Calculated.	N 6.63,	S 15.66.
Found.	" 6.78, 6.81;	" 15.68, 15.35.

7. Phenylacetyl Benzyl Cysteine.



Great difficulty was experienced in the preparation of this substance, and though sufficient amounts of a very pure product were obtained, still the means of isolation are as yet far from satisfactory. The synthesis was effected by means of the Schotten and Baumann reaction. Benzyl cysteine was dissolved in dilute sodium hydroxide solution and placed in a suitable bottle. To this was added in small amounts, about $1\frac{1}{2}$ molecules of phenylacetyl chloride for each molecule of benzyl cysteine. The container was shaken vigorously after each addition of the acid chloride, and the contents were kept *faintly alkaline* with sodium hydroxide. The operation lasted about an hour and a half.

At the end of this time, the material was transferred to a separatory funnel and acidified to Congo red with sulfuric acid. A yellowish, gummy, sticky precipitate appeared. This was taken up in several liters of boiling water, and the solution was then allowed to cool *very gradually*. After several hours there appeared some bunches of long, fine, white, crystalline needles, together with relatively large amounts of a yellowish brown, semifluid, amorphous material. The crystals were separated very carefully, dried, and tested for solubilities, melting point, and nitrogen content. According to the Kjeldahl method, they contained the theoretical percentage of nitrogen. They melted very sharply at 87–89°C. Evidently, therefore, they were neither benzyl cysteine nor phenylacetic acid. They were found, moreover, to be extremely soluble in alcohol, ether, carbon tetrachloride, benzene, acetone, ethyl acetate, and glacial acetic acid, as well as in alkalies. They were very insoluble, however, in

petroleum ether, carbon disulfide, and mineral acids. Coincidently, phenylacetic acid is similarly soluble in the same organic solvents, and is likewise insoluble in petroleum ether, but is very soluble in carbon disulfide. Benzyl cysteine, on the other hand, is remarkably insoluble in all of these organic solvents except glacial acetic acid.

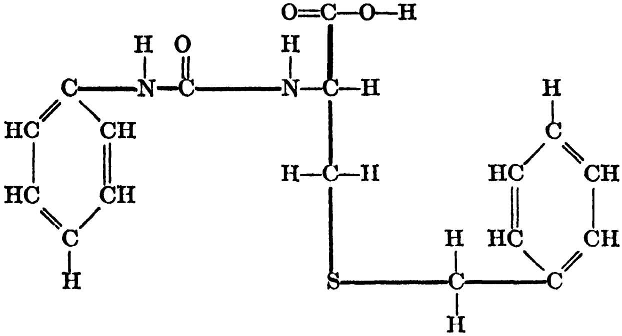
We were able, therefore, to obtain crystals of the pure material only by taking the mixture up in large amounts of boiling water and allowing the solution to cool very gradually. Crystallization was aided by impregnating the solution with a few crystals of the pure substance previously obtained. About a 25 per cent yield was realized on this first attempt.

Upon attempting the synthesis a second time, we first extracted, after acidification, with carbon disulfide to remove the phenylacetic acid. We then took the residue up in glacial acetic acid, filtered, and very carefully diluted the filtrate with water. The same unfavorable results, however, as recorded above, occurred; namely, the material precipitated as a yellowish brown gum. Accordingly, we finally took this gum up in a large amount of boiling water (about 6 liters for every 5 gm. of the initial benzyl cysteine), and allowed the solution to cool very slowly. We thus obtained several grams of the pure compound, together with rather large amounts of a more or less impure product. Some of this latter material, which appeared to be less impure and even to show signs of incipient crystallization, was collected and dissolved in glacial acetic acid. Upon carefully diluting the solution with water, we succeeded in obtaining a few grams more of the pure, crystalline product. The yield of this second trial, though a bit better than that of the first, was still poor—not over 35 per cent of the theoretical.

The substance dried at 50° *in vacuo* gave the following analysis by the Kjeldahl method.

Calculated.	N 4.26,	S 8.16.
Found.	" 4.03, 4.12;	" 7.92, 7.94.

8. Phenyluramino Benzyl Cysteine.



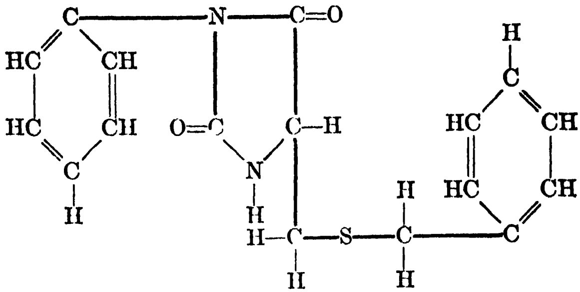
The benzyl cysteine (3 gm.) was dissolved in as small an amount of dilute sodium hydroxide solution as possible and placed in a 100 cc. bottle. To this were added 3 cc. of phenylisocyanate; *i.e.*, about 2 molecules for each molecule of benzyl cysteine. The container was then shaken vigorously for about 15 minutes, during which time it was cooled now and then under the tap. When the shaking was completed, the solution was diluted by the addition of about an equal volume of water, filtered by suction, and acidified to Congo red with hydrochloric acid. A very heavy, curdy, white precipitate formed at once. This was filtered, washed well with water, and taken up in acetone. As both benzyl cysteine and diphenylurea are insoluble in acetone they would have been removed by this operation. Finally, the filtered acetone solution was warmed and carefully diluted with water until a permanent cloudiness appeared. On cooling, white, feathery crystals separated, which, when dried in the oven at 60°C. for several days, melted at 145–146.5°C. The yield was apparently quantitative; namely, about 5 gm.

In alcohol and in ethyl acetate the substance dissolved quite readily in the cold and very easily on warming. It was fairly soluble in ether. Cold glacial acetic acid did not dissolve it, but the hot solvent did so to a small extent. In both cold and hot benzene, carbon tetrachloride, mineral acids, and water the substance was very insoluble.

The substance dried at 60° *in vacuo* gave the following analysis by the Kjeldahl method:

Calculated.	N 8.48,	S 9.69.
Found.	" 8.69, 8.56;	" 9.58, 9.71.

9. Benzyl Cysteine Phenylhydantoin.



5 gm. of benzyl cysteine phenylhydantoic acid were placed in a small beaker. Then about 75 cc. of 10 per cent hydrochloric acid were poured over the substance, and the beaker was placed on the water bath. After a couple of hours the material was apparently still unchanged, and seemingly none of it had dissolved. Accordingly, small amounts of acetone were added at intervals, until about 25 cc. had been added in all. At this stage most of the compound had dissolved. The heating was then continued for another couple of hours, after which the material was allowed to cool over night. The solid matter was then removed by filtration, taken up in about 25 cc. of *hot* 95 per cent alcohol, and filtered *very rapidly* by suction. On cooling, a very copious precipitate of bunches of fine needles separated. These were filtered, repurified a couple of times by recrystallization from hot alcohol, and dried in the oven. They melted quite sharply at 118–119.5°C.

The first mother liquor, after filtration, was diluted with about an equal volume of water, and placed in the ice box. After a couple of days another copious yield of the same crystals was obtained. The complete yield was about 85 per cent.

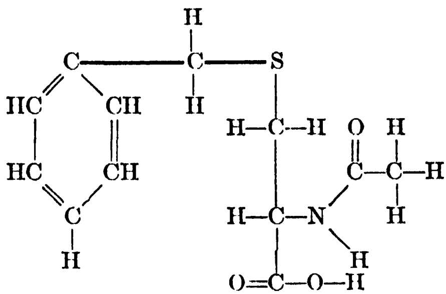
The material was very soluble in cold ethyl acetate, benzene, and especially acetone. From acetone it precipitated on dilution with water. When the benzene solution was heated, a pretty lavender or purplish color developed, which turned to a permanent blue or greenish blue on cooling. In carbon tetrachloride and in ether the substance dissolved somewhat in the cold, but readily on warming. It was very slightly soluble in cold glacial acetic acid, and not much more so in the hot solvent.

In cold and hot water, however, mineral acids, and alkalies, it was very insoluble.

Dried at 60° *in vacuo* the analysis by the Kjeldahl method was as follows:

Calculated.	N 8.97,	S 10.24.
Found.	" 8.88, 9.01;	" 10.07, 10.16.

10. Acetyl Benzyl Cysteine.



We adapted to this synthesis the principle employed by Friedmann (5) in the acetylation of *p*-bromophenyl cysteine.

The benzyl cysteine, of which 10 gm. were used, was pulverized and placed in a 250 cc. Erlenmeyer flask. About 100 cc. of pyridine were then introduced and the mixture was well stirred. Acetyl chloride was then added in small amounts until the benzyl cysteine had practically all dissolved. After each addition of the acid chloride the container was vigorously shaken, and was kept well cooled by tap water. The mixture was then allowed to stand in the cold for several hours, after which it was strongly acidified with concentrated hydrochloric acid, and allowed to stand for another several hours. No crystals or precipitate of any kind appearing, the mixture was filtered, made alkaline with sodium hydroxide, transferred to a separatory funnel, and extracted twice with about 150 cc. of ether each time. After allowing the liquids to settle, the lower layer (the water solution), was drawn off, filtered, and acidified to Congo red with hydrochloric acid. A dense cloudiness appeared at once, and on rubbing the sides of the beaker briskly with a glass rod for several minutes a copious, crystalline precipitate separated. After standing in the cold for several hours, this was filtered off, washed

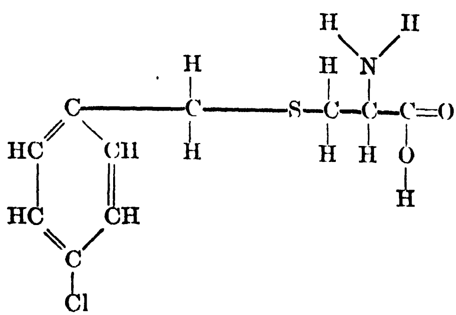
well with cold water, dissolved in a small amount of hot 95 per cent alcohol and poured into about 700 cc. of *moderately* hot water (not over 75°C.). On cooling, a plentiful precipitate of fine, white needles appeared. These were removed by filtration, washed well with cold water, and dried in the oven at 80°C. for several days. They then melted at 156–157°C.

After allowing the funnel containing the ether-pyridine mixture to stand for several days, the two materials separated into two layers. The lower one, containing the pyridine, was then drawn off and acidified strongly with concentrated hydrochloric acid. Immediately, a very heavy precipitate appeared. After standing for several hours in the cold, this was filtered off, washed well with cold water, and treated in the same way as described above. A second yield of the same substance was thus obtained. The total yield was about 50 per cent.

The compound was quite soluble in hot water, but very insoluble in cold water. In glacial acetic acid, ethyl acetate, and acetone, solution occurred with difficulty in the cold, but more easily on warming. Ether, benzene, and carbon tetrachloride, both cold and hot, did not effect solution. Acid hydrolysis of the substance yields acetic acid and benzyl cysteine.

The substance contained, according to Kjeldahl, 5.65 and 5.70 per cent nitrogen as compared to the theoretical amount of 5.53 per cent, while sulfur was calculated as 12.64 per cent and found on analysis to be 12.52 per cent.

11. p-Chlorobenzyl Cysteine.



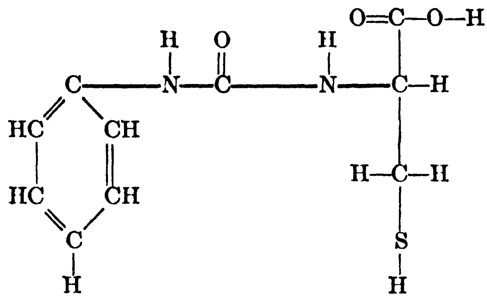
In this synthesis exactly the same procedure was followed as in the preparation of benzyl cysteine, except that *p*-chlorobenzyl

chloride was used instead of benzyl chloride. The final purified product, when dried in the oven at 80°C. for several days, melted at 219–220°C.—a trifle higher than the benzyl cysteine. It had, moreover, the same solubilities as the benzyl cysteine. The synthetic reaction, however, seemed to be a bit slower and more difficult in the case of the *p*-chloro compound, with the result that a somewhat lower yield was realized.

Analysis, after drying at 80°C., gave the following results.

Calculated.	N 5.69,	S 13.00.
Found.	" 5.86, 5.81;	" 13.14, 13.27.

12. Phenyluramino Cysteine.



To effect this synthesis, 5 gm. of cysteine hydrochloride were dissolved in about 100 cc. of water and placed in a 250 cc. bottle. To this were added 10 gm. of phenylisocyanate, and about 9 cc. of 50 per cent sodium hydroxide solution. The bottle was then shaken vigorously for 10 or 15 minutes, being kept well cooled the while by tap water. The contents were then filtered by suction and acidified to Congo red with dilute hydrochloric acid. At once, a heavy, white, curdy precipitate formed. This was filtered, thoroughly washed with water to remove all acid, sodium chloride, and any uncombined cysteine, and dried well in the oven at 70°C. It was then dissolved in about 25 cc. of ethyl acetate and filtered, to remove any phenyluramino cystine which might have formed during the interaction. The ethyl acetate was then evaporated by directing a current of warm air by means of an electric fan over the evaporating dish. After the odor of the ethyl acetate had practically disappeared and only a thick syrup remained, the material was taken up in about

100 cc. of 95 per cent alcohol. This solution was then carefully diluted with water until a permanent milkiness appeared. Within a few minutes the substance crystallized out in very fine, short, microscopic, white needles, very different from phenyluramino cystine, whose crystals appear as long, stringy feathers. After allowing the crystals to stand in the ice box for several hours, they were filtered, washed well with 5 per cent alcohol, and dried first by air and then *in vacuo* at 75°C. for 12 hours. They then melted quite sharply at 134–136°C.

The substance gave a very strongly positive nitroprusside reaction—a reaction which is negative for cystine and for phenyluramino cystine. It was exceedingly soluble in ethyl acetate, and moderately soluble in ether—unlike phenyluramino cystine. According to the Kjeldahl method, it contained the proper percentage of nitrogen.

1 gm. of the material was dissolved in dilute sodium hydroxide solution and shaken vigorously for about half an hour with 2 cc. of benzyl chloride. A product was thus obtained which melted at 145–146.5°C., the melting point of phenyluramino benzyl cysteine, previously prepared by us (*cf.* above). This product was then treated to form the hydantoin, with the result that a substance was obtained which melted at 118–120°C.

The phenyluramino cysteine was found to be very soluble in alcohol, from which it crystallized on dilution with water. It was similarly soluble in acetone, but on dilution with water precipitated as a gum. It was extremely soluble in ethyl acetate, and on evaporation of the solvent remained as a thick syrup. It was moderately soluble in ether. In carbon tetrachloride, however, carbon disulfide, and benzene, both cold and hot, it was very insoluble. The yield was about 80 per cent.

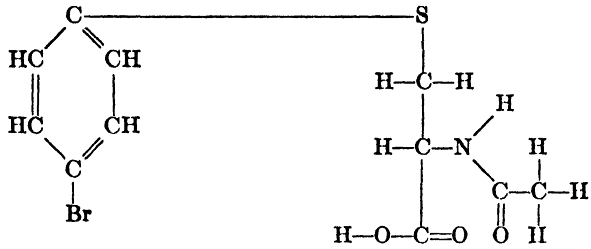
Though insoluble in *cold* glacial acetic acid and in *cold* water, the substance seemed to be quite soluble when the solvents were heated to about 95°C. However, there was much evidence, both in these cases and in others, that under such conditions the substance decomposed. Apparently, therefore, at this temperature it is stable only when dry.

We have not yet prepared the hydantoin of this compound, but are working in that direction.

Analysis of the substance, after drying *in vacuo* at 75°, gave the following results.

Calculated.	N 11.67,	S 13.33.
Found.	" 11.80, 11.92;	" 13.68, 13.72.

13. p-Bromophenyl Mercapturic Acid.



The simplest means to obtain this compound seemed to be the employment of the organism of the dog (6). Accordingly, a fairly large dog of about 15 kilos body weight was placed in a roomy metabolism cage, and fed with food rich in protein, together with a 6 gm. dose of bromobenzene—the latter by means of a stomach tube. The animal was then kept in the cage for at least 36 hours. At the end of this time he was replaced by another animal, and the process was repeated.

The 36 hour collection of urine from each feeding was filtered quickly through glass wool, acidified strongly by the addition of about one-third its volume of concentrated hydrochloric acid, and allowed to stand in the ice box for about 10 days. We found that this was ample time for all the mercapturic acid to crystallize out. After a dozen or so samples of urine had been thus prepared, the solid matter was filtered off and dissolved in 5 per cent ammonium hydroxide. After refiltering, it was decolorized with good animal charcoal, filtered, and acidified strongly to Congo red with hydrochloric acid. After standing in the cold for several hours, the bromophenyl mercapturic acid precipitated completely as fine, short, slightly grayish needles (M.P. 152–153°C.). The yield was about 25 gm. from 100 gm. of bromobenzene.

The compound is soluble in hot water to the extent of about 1 part in 70. It is also quite soluble in alcohol, carbon tetrachloride, and benzene. It is very insoluble in cold water and in ether.

SUMMARY.

For purposes connected with our work regarding the metabolism of cystine, we prepared the following substances: cystine, diphenylacetyl cystine, phenyluramino cystine, cystine phenylhydantoin, cysteine hydrochloride, benzyl cysteine, phenylacetyl benzyl cysteine, phenyluramino benzyl cysteine, benzyl cysteine phenylhydantoin, acetyl benzyl cysteine, *p*-chlorobenzyl cysteine, phenyluramino cysteine, and *p*-bromophenyl mercapturic acid. Of these compounds, diphenylacetyl cystine, phenylacetyl benzyl cysteine, phenyluramino benzyl cysteine, benzyl cysteine phenylhydantoin, *p*-chlorobenzyl cysteine, acetyl benzyl cysteine, and phenyluramino cysteine had not been prepared before. Accordingly, we studied these latter compounds in more or less detail.

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STUDIES OF THE CARBON DIOXIDE ABSORPTION CURVE OF HUMAN BLOOD.

I. THE APPARENT VARIATIONS OF pK_1 IN THE HENDERSON-HASSELBALCH EQUATION.

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In 1916 Hasselbalch (1916–17) demonstrated experimentally what L. J. Henderson (1908) had already deduced theoretically, that the hydrogen ion concentration of blood could be calculated from the carbon dioxide tension and the bicarbonate concentration with a mean error no greater than that of the electrometric method, which he estimated to be about ± 0.02 pH. For convenience he transformed Henderson's equation

$$C_H = K \frac{[H_2CO_3]}{[BHCO_3]} \tag{1}$$

into the logarithmic form

$$pH = pK_1 + \log \frac{[BHCO_3]}{[H_2CO_3]} \tag{2}$$

Hasselbalch found that in bicarbonate solutions pK_1 varied with the concentration of bicarbonate and assumed that it varied in the same manner in blood. Milroy (1917), Parsons (1919–20), and Michaelis (1920) concluded that there were no theoretical grounds for the assumption of such a variation in blood and in their studies assumed a constant value for pK_1. Their position has been accepted by most workers.

Warburg (1922) has shown that the views of Hasselbalch and of Parsons are not irreconcilable. He has reviewed and analyzed all the previous work on blood bicarbonate and hydrogen ion

concentration and has added data of his own obtained by methods more accurate than those of previous workers. He points out that the constant, pK_1, in the Hasselbalch equation does not vary with the bicarbonate concentration in blood; but that the volume of the blood corpuscles and the partition of CO_2 between the corpuscles and the plasma vary according to the pH of the blood and cause corresponding variations in the ratio $\frac{[BHCO_3]}{[H_2CO_3]}$ which produce apparent changes in pK_1. He shows that Parsons' values for pK_1, treated statistically, show a consistent variation with varying pH that is outside the limits of error of his methods.

All these studies have depended on a comparison of electrometric measurements of the hydrogen ion concentration and determinations of the carbon dioxide content of blood which has been brought into equilibrium with gas mixtures of known carbon dioxide tension. It is, however, impossible to settle the question satisfactorily by such comparisons. In a complex biological system such as blood, or even plasma, one cannot hope to attain an accuracy greater than $\pm$ 0.01 to 0.02 pH by electrometric measurements. This corresponds to a difference of $\pm$ 1 to 2 volumes per cent of CO_2. But by the latest improved methods of saturation and analysis of gases in the blood this error has been reduced to $\pm$ 0.2 to 0.5 volume per cent of CO_2 (Austin et al., 1922; Van Slyke and Stadie, 1921). The changes which might be expected to occur in pK_1 are hardly beyond the limits of error of the electrometric method, but should be easily demonstrable by gasometric methods.

EXPERIMENTAL.

In the course of some studies of electrolyte equilibria in the blood, certain data have become available that permit an evaluation of the variations of pK_1 in whole blood. In one series of experiments oxalated whole blood, obtained from patients in the medical wards of the New Haven Hospital and from members of the hospital and laboratory staff, was saturated with known tensions of CO_2 in air. One portion of the blood, thus saturated, was analyzed for CO_2 and the volume of its cells was determined by hematocrit. From another portion the plasma was separated and analyzed for CO_2. The oxygen capacity of the blood was

also determined. 68 determinations were made on 56 specimens of blood. The experiments fall into two groups. In forty-four experiments the blood was studied at 40 mm. CO_2 tension only; in twelve instances determinations were made at both 30 and 60 mm.

Methods.

Saturation with CO_2 was effected by the method described by Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (1922) as "Second saturation method," with only slight variations. The tonometers used held 700 to 800 cc. and the greatest amount of blood used at one time was 25 to 30 cc. Under these conditions it was found unnecessary to correct for the CO_2 given off or taken up by the blood. Each sample was exposed in two successive tonometers to a gas mixture calculated to give the exact CO_2 tension desired after it had come to the temperature of the water bath (38°C.) in which it was rotated. In order to make sure that this method effected complete equilibrium a third saturation was done in a series of experiments. In no case was there any change in the carbon dioxide content of the blood after the second saturation when as much as 30 cc. of blood were exposed to 20 or 40 mm. of CO_2 tension as is shown in Experiment 1.

We have preferred the "Second saturation method" to the "First," because it is simpler and permits the production of the exact CO_2 tension desired, and because we have not been able to convince ourselves by theory or experiment that the "First" is more accurate. To be sure there is no possibility of checking the gas mixture in the "Second method" and the gas mixture must be prepared with great accuracy. In only one experiment of a much longer series than that here presented, is there any internal evidence of an error in the preparation of the original gas mixture. On repeated occasions duplicate blood samples exposed in different tonometers have shown no greater variation than duplicate samples from the same tonometer, an indication that no significant errors enter in the preparation of gas mixtures. (See Experiment 2.) Attempts to analyze the gas mixtures disclosed certain unforeseen difficulties. In the manifold described (Austin et al., 1922) the CO_2 is introduced from a carefully calibrated and water-jacketed burette at known temperature, and saturated with water vapor at atmospheric pressure. The diluent, air, is allowed to enter from the room at a different and variable temperature through a small amount of water. When the gases are analyzed in the Haldane burette it is assumed that both CO_2 and diluent were introduced, saturated with water vapor, at the same temperature and pressure. In testing out the method the fact that such an assumption was involved at first escaped recognition, and unaccountable differences were found between the calculated and observed CO_2 tensions in the tonometers. When precautions were taken to ascertain the pressure and temperature of the diluent air and to insure its saturation with water vapor these differences at once disappeared. (See

Experiment 3.) The total pressure in the system is not important in these experiments provided the final oxygen tension is sufficient to insure complete oxygen saturation and this is amply provided for in either method. The "First saturation method" attempts to overcome these difficulties by opening the tonometers to the air in the course of saturation and thus assuring equilibrium with atmospheric pressure. This renders it impossible to obtain the exact CO_2 tension desired. The method which we have chosen is not as well adapted to experiments that demand the establishment of exact pressure conditions for more than one gas because it presupposes that all the gases under investigation are pure. That this assumption is justified when CO_2 is obtained from a Kipp generator with a water trap to remove acid, as it was in our experiments, we have proved by analysis; but it is not justified when one is dealing with gases obtained from commercial cylinders.

It was shown by Christiansen, Douglas, and Haldane (1914) and has since been emphasized by Dale and Evans (1922) that the CO_2 capacity of blood diminishes progressively if it is permitted to stand. Austin et al. (1922) found that this change was very slow in horse blood and could be eliminated by chilling the blood. We have determined the rate of change in human blood and have found that, if blood is kept chilled (standing in water drawn from an ice cooler), its CO_2 capacity remains unchanged for at least 3 hours. This is true even when it is left with a considerable surface exposed to the air, in a thin layer in the bottom of a Pyrex glass beaker. (See Experiment 4.) All specimens of blood used in these experiments were kept chilled in narrow tubes. The blood, however, was saturated as soon as possible (15 to 60 minutes) after it was drawn.

Experiment 1.—30 cc. of blood were exposed in two successive tonometers to CO_2-air mixtures containing the proper amount of CO_2 to give the desired tension at 38°C. 5 cc. were then removed for analysis and the remaining 25 cc. exposed in a third tonometer to the same tension of CO_2. This blood was then analyzed for CO_2.

CO_2 tension.	CO_2 content		
	After second saturation	After third saturation.	Difference.
mm.	*vol per cent*	*vol per cent*	*vol per cent*
20	42.3	42.5	0.2
20	24.5	24.3	0.2
40	60.1	60.0	0.1
40	61.9	62.1	0.2

Experiment 2.—Duplicate samples of blood were saturated in separate tonometers, delivered over mercury, and then analyzed for CO_2 in duplicate.

Experiment No.	CO_2 tension.	CO_2 content.		
		Tonometer 1.	Tonometer 2.	Difference.
	mm.	*vol. per cent*	*vol. per cent*	*vol. per cent*
1	40	58.6	58.6	0.0
2	40	55.0	55.0	0.0
3	40	59.9	60.0	0.1

Experiment 3.—CO_2-air mixtures were introduced into tonometers containing a small amount of water, in the manner described in the "Second saturation method." Samples of the gas were then analyzed in a Haldane gas burette and the CO_2 tension was obtained by analysis compared with that calculated by formula from the size of the tonometer, the amount of CO_2 introduced, the amount of air introduced, and the temperature and pressure of both CO_2 and air.

CO_2			CO_2		
Calculated.	Observed.	Difference.	Calculated.	Observed.	Difference
vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
4.92	4.99	0.07	5.34	5.27	0.07
4.32	4.27	0.05	4.84	4.80	0.04
4 93	4.83	0.10	4.89	4.81	0.08

Experiment 4.—Blood was withdrawn from the arm vein of J.P. at 10.30 a.m. into a tube containing potassium oxalate. It was then divided into two equal portions. One was kept over mercury, without contact with air, in the tube into which it was originally drawn. The other half was placed in an open beaker. Both samples were chilled at once. The sample in the open beaker was stirred thoroughly.

A sample from each specimen was saturated at once at 38° with 40 mm. CO_2. (Saturation complete at 11.20 a.m.) The remainder of each was allowed to stand chilled until 2 p.m. when samples were again taken and analyzed.

Determination No.	Results of analysis:	CO_2
		vol. per cent
1	Saturated at once, without contact with air................	63.0
2	" " " after preliminary exposure and shaking.	63.3
1a	" " 2 p.m. without contact with air..............	63.1
2a	" " 2 " after exposure to air in beaker........	63.5

The time of exposure in the 38° water bath was 15 minutes for each saturation. Experiments indicated that 10 minutes were sufficient to effect equilibrium and that 15 minutes were not enough to produce any alteration in the CO_2 capacity of the blood.

After saturation the blood was transferred to sampling tubes over mercury and was centrifugated in completely filled stoppered tubes, from which the plasma was removed to similar sampling tubes with all the precautions to avoid loss of CO_2 described by Austin et al. (1922).

The analyses of blood and plasma for CO_2 were performed in the manner described by Van Slyke and Stadie (1921) in a Van Slyke burette, of the long stem type, graduated in 0.01 cc., water-jacketed and provided with a mechanical shaker (Stadie, 1921). The burettes were calibrated by weight of mercury delivered as suggested by Y. Henderson (1918) for the calibration of Haldane burettes. The calibration was further verified by the analysis of freshly prepared solutions of anhydrous sodium carbonate.

Van Slyke and Stadie (1921) have pointed out that there is a slight reabsorption of CO_2 by the small amount of fluid left in the Van Slyke burette when atmospheric pressure is restored to permit measurement of the gas. Van Slyke obtained only 98.2 per cent of theory in the analysis of carbonate solutions and has introduced a corresponding correction into his last equation for the calculation of CO_2. We have determined this error in a number of Van Slyke burettes and have found that it is constant for any one apparatus, but varies in different instruments. We have, therefore, determined the extent of the error empirically for each instrument and introduced a corresponding correction. Two burettes have been employed in this series of experiments: one has a correction factor of $\frac{1}{0.986}$, the other of $\frac{1}{0.977}$. These factors have been verified by three observers to eliminate the possibility of personal variations.

The mean variation in duplicate determinations by this method is less than 0.2 volume per cent and the agreement between specimens exposed in different tonometers has been equally good.

The effect of oxalate on the CO_2 capacity of blood has been a continual source of controversy. Austin et al. demonstrated that in concentrations of less than 0.5 per cent it did not alter the CO_2 capacity of blood. Warburg (1922) agrees with this but adduces evidence that oxalate alters the distribution of CO_2 between the plasma and cells. In the same experiments by Austin et al., however, the CO_2 capacities of plasma from both defibrinated and oxalated blood also were found to be identical.[1] These experiments were carried out with the utmost care with the express purpose of determining the effect of oxalate. In a concentration of 1 per cent, potassium oxalate does alter the CO_2 capacity, but it also produces hemolysis in most specimens of blood. In concentrations of less than 0.5 per cent we are convinced that no such changes occur. In all our experiments

[1] Unpublished data.

the blood was drawn into tubes containing just enough neutral potassium oxalate, crystallized on the walls of the tubes, to give a final oxalate concentration of not less than 0.2 per cent and not more than 0.4 per cent.

The oxygen capacity was determined by a modification of the method of Van Slyke and Stadie devised by Dr. C. Lundsgaard,[2] in which both saturation and analysis are performed in the Van Slyke burette. The method gives results identical with those obtained by the older methods.

Hematocrit determinations were made by means of the ordinary Daland hematocrit fitted to a No. 1 International Equipment Company centrifuge. Duplicate determinations were made on each blood sample and only two pairs of hematocrit tubes were used throughout. Centrifugation at high speed was continued until the cells were transparent. Duplicates varied by less than 1 per cent. Although the absolute values obtained by any hematocrit method are open to question, the relative values here reported are sufficiently accurate for our purposes.

Parsons (1919–20) has pointed out that whether blood or plasma is used for electrometric determination of pH, the value obtained represents the hydrogen ion concentration of the plasma only. In this view Warburg (1922) concurs. It follows naturally that, if proper values for pK_1 are assumed, the pH values calculated from plasma and blood by the Hasselbalch equation, should agree. Van Slyke (1922) estimated the value of pK_1 in plasma and blood as 6.100 and 6.150, respectively. However, when the pH of plasma and that of whole blood are calculated with these pK_1 values no agreement is obtained, as is shown by Columns 11 to 13 of Table III. If there were a constant difference between pK_1 of blood and plasma of 0.05, the values in Column 13 should be constant. On analysis, however, the values are seen to increase with the hemoglobin or cell content of the blood. More careful study reveals the fact that there is a lesser tendency for the difference to vary with pH. These variations are of the same kind as those obtained by Warburg. That there is a valid basis for such an apparent variation of pK_1 Warburg has shown in a careful theoretical and mathematical analysis of the various factors involved in the determination of pH by the Henderson-Hasselbalch equation. There is, according to Warburg, no change in pK_1, itself, for such a change would involve a variation in the dissociation constants of carbonic acid and bicarbonate. There is, however, a change in the factors that go to make up the ratio

[2] Personal communication.

$\frac{[BHCO_3]}{[H_2CO_3]}$. If the equation is written in logarithmic form it is clear why such corrections cause an apparent change in pK_1.

$$pH = pK_1 + \log [BHCO_3] - \log [H_2CO_3] \qquad (3)$$

If we assume a correction factor for $[BHCO_3]$, m, and one for $[H_2CO_3]$, n, this equation will read:

$$pH = pK_1 + \log [BHCO_3] + \log m - (\log [H_2CO_3] + \log n) \qquad (4)$$

These two corrections can be combined with pK_1.

$$pH = (pK_1 + \log m - \log n) + \log [BHCO_3] - \log [H_2CO_3] \qquad (5)$$

or

$$pH = pK'_1 + \log [BHCO_3] - \log [H_2CO_3] \qquad (6)$$

If pK_1 in plasma is constant, the pH of plasma can be determined from the CO_2 content of whole blood at known tension if some means can be devised to calculate the $[BHCO_3] : [H_2CO_3]$ ratio in plasma from that in whole blood. By the mathematical treatment of data collected from the literature and from experiments of his own, Warburg derived an equation by which he believed the $[BHCO_3] : [H_2CO_3]$ ratio and therefore the pH of plasma could be calculated from the CO_2 content, CO_2 tension, and hemoglobin concentration of whole blood. He also presents a graphic chart to facilitate the calculation. Warburg's correction factors were developed largely from the results of experiments on horse blood at room temperature. On the basis of a few experiments on human blood at 38°C. and the data of Joffe (1920–21) he concluded that horse blood at room temperature was very similar to human blood at body temperature in its reaction to CO_2. When Warburg's correction factors were applied to our experiments the results were highly unsatisfactory. Furthermore, an attempt to derive a new set of correction factors by means of Warburg's equation proved unsuccessful. A graphic chart (Chart 4) has, however, been constructed which permits the calculation of plasma pH from blood CO_2. Our inability to utilize Warburg's equation for the development of correction factors does not necessarily imply any error in the principles on which this equation was developed. The mathematical develop-

ment of the equation is unquestionably logical; but certain assumptions which Warburg has made with regard to the variables in the equation are not in agreement with the experimental values here presented. In order to make this clear it is necessary to consider certain aspects of Warburg's theory, but it is not necessary, for our purposes, to follow all the steps of his mathematical treatment.

In brief the problem which presents itself is to predict the $[BHCO_3]$: $[H_2CO_3]$ ratio of plasma from that of blood. In this ratio $[H_2CO_3]$ is calculated as dissolved CO_2 by the equation

$$\frac{\text{p } CO_2 \, \alpha}{760} = [H_2CO_3] \qquad (7)$$

in which α is the solubility coefficient, and

$$[BHCO_3] = \text{Total } CO_2 - [H_2CO_3] \qquad (8)$$

Bohr (1905) found the relative solubility coefficient for CO_2 in plasma to be 0.975, so that $\alpha_{\text{plasma}} = 0.975 \; \alpha_{\text{water}}$. The relative solubility coefficient for CO_2 in whole blood was somewhat lower and has been fixed by most workers at 0.91 to 0.92. Warburg points out that it is not proper to use a mean solubility coefficient for the calculation of the dissolved CO_2 in blood. Blood is a mixture of cells and plasma and the solubility coefficient in blood will depend on the relative amounts of plasma and cells present.

According to Bohr, the relative solubility coefficient for CO_2 in red blood cells is 0.81. By applying this value and using hematocrit determinations to measure the relative amounts of cells and plasma one should obtain a more exact solubility coefficient for whole blood. For our purposes, in which all work is done at constant temperature, a simple equation has been devised.

$$\begin{aligned} \alpha_{CO_2 \text{ (water)}} \text{ at } 38°\text{C.} &= 0.555 \text{ (Bohr)} \\ \alpha_{CO_2 \text{ (plasma)}} \text{ at } 38°\text{C.} &= 0.975 \times 0.555 \\ \alpha_{CO_2 \text{ (blood cells)}} \text{ at } 38°\text{C.} &= 0.810 \times 0.555 \end{aligned}$$

The amount of CO_2 dissolved in a unit of blood is

$$0.555 \, \frac{p_{CO_2}}{760} \, [0.975 \text{ (volume of plasma)} + 0.81 \text{ (volume of cells)}] = [H_2CO_3] \qquad (9)$$

If we call the volume of cells, c, the volume of plasma = 1–c and we get

$$\frac{0.555}{760}\{0.975\,(1-c)+0.81\,c\}\,p_{CO_2}=[H_2CO_3]$$

This can be simplified to

$$(0.7118-0.1205\,c)\,p_{CO_2}=[H_2CO_3] \qquad (10)$$

By means of Chart 1 the CO_2 dissolved in blood with different proportions of cells can be obtained at sight.

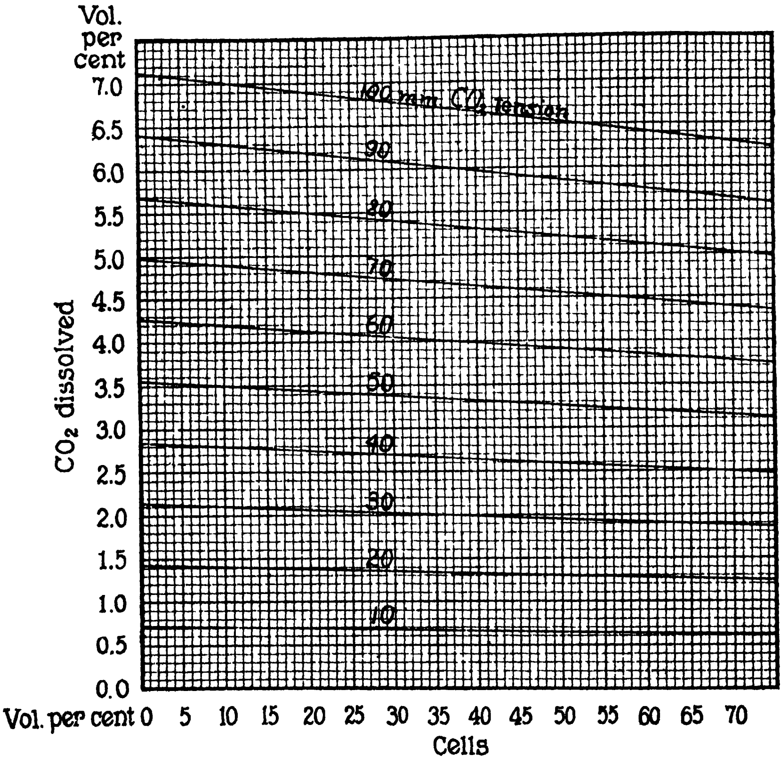

Chart 1. For the determination of CO_2 dissolved in blood with varying cell volume. Oxygen capacity divided by 0.45 may be used instead of cell volume.

Warburg estimates the average ratio of oxygen capacity to cell volume in human blood at 0.48. Our own determinations fix this ratio at 0.465 at an average pH of 7.3. Our values agree better

than Warburg's with other estimates found in the literature. By dividing the oxygen capacity by 0.465 one can obtain the corresponding value for cell volume and this value can be used to derive the dissolved CO_2 from Chart 1 or equation (10).

The true value of $[BHCO_3]$ in blood is obtained by subtracting from the total CO_2 found the $[H_2CO_3]$ value indicated by Chart 1. It remains to find the relation between the $[BHCO_3]$ of whole blood and that of plasma. This relation is defined by Warburg's equation

$$[BHCO_3]_{(plasma)} = [BHCO_3]_{(blood)} \times \frac{100}{100 - Q\ (1 - D)} \qquad (11)$$

where Q = the cell volume in volumes per cent and D = the ratio $\frac{[BHCO_3]_{(cells)}}{[BHCO_3]_{(plasma)}}$. In this equation D is unknown and it remains to determine it or to see how closely it can be predicted. Warburg considers that the pH of the blood is the chief determinant of the value of D and he has determined the relation of D to pH. That D varies with pH in our experiments, also, is evident from Chart 2 in which the abscissa represents the pH of plasma and the ordinate the reciprocal of D. (We have used the reciprocal of D instead of D because it gives a better distribution of points and brings the relation out more clearly.) The deviations from the mean line, AB, are, however, considerable and suggest that there are variable factors other than the pH which influence the value of D.

Many of the experiments of this series were done on blood from patients with diseases which produced obvious changes in other substances in the blood than CO_2. It seemed possible that the scattering was due to such pathological changes. One or two of the extreme deviations seemed to support this view, but the remainder showed little more scattering among pathological bloods than among normal bloods. A comparison of the experiments in which only one point of the absorption curve was determined and those in which two points were determined revealed an interesting anomaly. The change in D observed in the two point experiments was always less than would have been expected if D varied according to the statistical average AB, derived from the mean of the whole group of the experiments. This is shown in Chart 2

where the changes in the two point experiments are represented by the fine lines. In no case is the slope of one of these lines as great as the slope of the line AB.

In studies of the respiratory function and gaseous equilibrium of the blood it is often more useful to know the oxygen capacity than the cell volume. If the oxygen capacity is to be used for the calculation of the difference between the [$BHCO_3$] of blood and that of plasma, instead of the hematocrit value, Q in equation

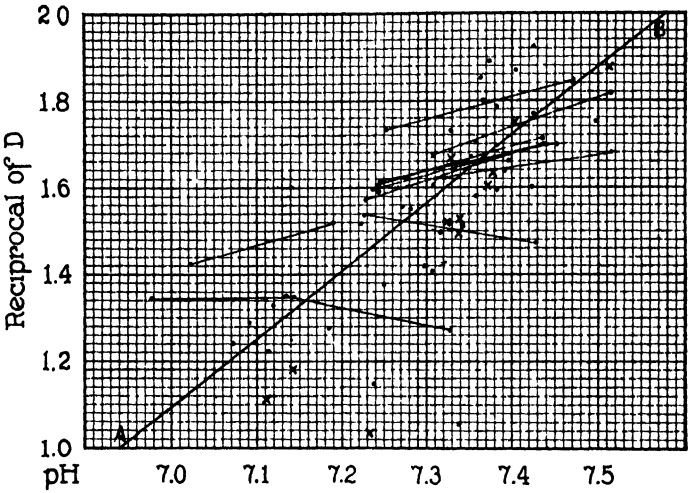

CHART 2. Abscissa = pH; ordinate = ratio of concentration of [$BHCO_3$] of plasma to that of cells.

AB = Average line relating distribution coefficient to pH.

. = Data from experiments of this series.

× = Additional data from other experiments.

The change in two point experiments is indicated by the fine lines.

(11) also becomes a variable. Hamburger (1902) demonstrated that the cell volume of blood was not a constant, but varied with changes in pH. This observation has been repeatedly confirmed and is evidenced in the two point experiments of this series. Warburg has studied the relation of cell volume to pH in horse blood and has substituted the values thus derived for Q in equation (11). These values he admits are not exactly applicable to human blood; but the error thus introduced he considers negligible.

Chart 3 shows the relation of hematocrit values to pH in our series of experiments. In this chart the abscissa represents pH, the ordinate the ratio of oxygen capacity to cell volume. Although statistical treatment shows a slight tendency for the cell volume to increase as the pH falls, the scattering is so great that no mean curve drawn from these data would be of any value. It seems hardly possible to ascribe these variations to errors in hematocrit

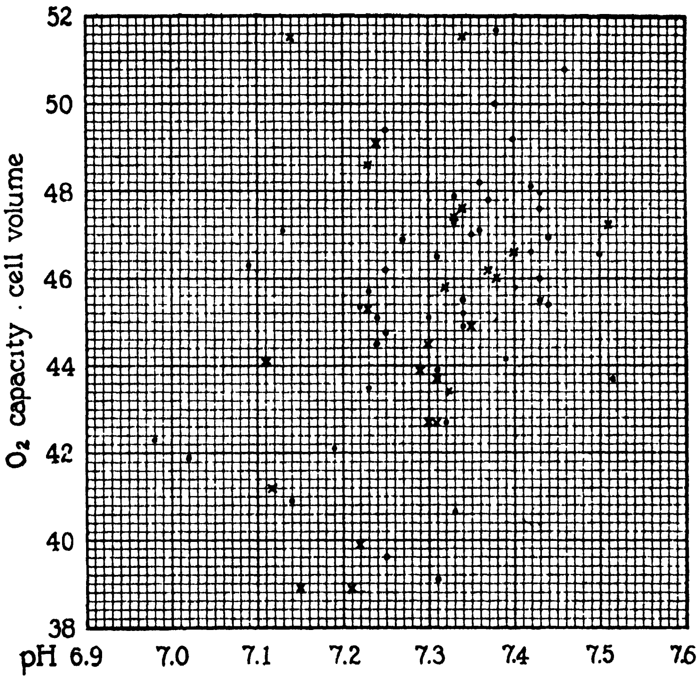

CHART 3. Abscissa = pH; ordinate = ratio, O_2 capacity: cell volume.
. = Data from experiments of this series.
× = Additional data from other series of experiments.

determinations alone, because in the two point curves the change in cell volume was consistently obtained.

If neither D nor Q in equation (11) can be predicted with certainty it is clear that Warburg's equation, though in itself correct, cannot be used for the calculation of the apparent difference between pK_1 of blood and that of plasma. It may be added that Warburg's correction factors gave a greater error than did his equa-

tion with mean values of Q and D derived from our data. This was to be expected because our values for Q and D are entirely different from his. It remained to devise empirical correction factors. The observed differences between log $\frac{[BHCO_3]}{[H_2CO_3]}$ in whole blood and in plasma, which we have called $\Delta\ pK_1$, do, as we have said, show a definite tendency to increase as the oxygen capacity increases and a lesser tendency to increase with increasing pH.

The relation of $\Delta\ pK_1$ to cell volume alone was first estimated by the method of group averages. The results are shown in

TABLE I.

Group No.	No. of points.	Range of cell volume variation.	Average cell volume.	Average pH.	Average $\Delta\ pK_1$.
		vol. per cent	*vol. per cent*		
I	4	14.7 to 19	15.9	7.141	+0.005
II	7	20 to 29	26.7	7.182	+0.016
III	26	30 to 39	36.8	7.276	+0.036
IV	31	40 to 49	43.6	7.308	+0.049

TABLE II.

Group No.	pH	No of points.	Average pH.	Difference between high and low pH averages.	Difference in average $\Delta\ pK_1$.	Change in $\Delta\ pK_1$ per 0.1 pH.
III	<7.3	11	7.167	0.189	0.012	0.006
	>7.3	15	7.356			
IV	<7.3	14	7.242	0.124	0.008	0.006
	>7.3	15	7.366			

Table I. There can be no doubt that $\Delta\ pK_1$ varies with the cell volume. Groups III and IV were then divided into two subgroups each, according as the pH lay above or below 7.3. The results are shown in Table II. Here again there is an unmistakable relation. The difference is, as far as can be judged from these figures, practically constant between cell volumes of 30 and 50. Below this there are not sufficient points to permit the analysis of the variation with respect to pH.

The mean values obtained for Groups III and IV in Table I were corrected by the corrections from Table II to a pH of 7.3,

and a straight line was drawn through these points from the cell volume line 30 to the cell volume line 50 (Chart 4). Lines parallel to this and 0.006 apart were then drawn to represent pH variations from 7.5 to 7.0. Below 30 volumes per cent of cells the number of observations were so small that any continuation of the lines was open to suspicion. The broken lines in Chart 4 were therefore drawn to conform to the values shown for Groups I and II in Table I and the pH corrections from Table II were continued. This undoubtedly involves an error; but the substitution of another correction value would have been purely arbitrary. (In order to make the upper portion of the curves meet the dotted prolongations it was necessary to curve the former somewhat. The alteration from the straight line form, however, at no point exceeds 0.001 pH.)

DISCUSSION.

Comparison of the observed values of Δ pK_1 and those obtained by means of Chart 4 is shown in the last three columns of Table III. The average deviation of all the experiments is $\pm$ 0 007. The average deviation of the 57 observations with cell volume of over 30 is the same. In the experiments with high cell volume the deviations are evenly scattered: the total of the negative deviations is 197 against 204 for the positive deviations. The calculated values in the experiments with low cell volume, on the other hand, show a definite tendency to lie too high.

Although the data available are too scanty to warrant an attempt to correct the lower portions of the curves, something of the probable nature of such corrections may be predicted. The [$BHCO_3$] concentration in the cells has never been observed to exceed the [$BHCO_3$] concentration in the plasma, if we except a few experiments by Haggard and Henderson (1920-21). Furthermore, all observers have agreed that the pH of the cell contents is lower than that of the plasma. That this should be the case is in keeping also with Donnan's (1911) equilibrium theory. But, if the pH and [$BHCO_3$] concentration are always lower in the cells than in the plasma, Δ pK_1 must always have a positive value. In order that this may be so, the differentials $\frac{d\Delta pK_1}{d\Delta pH}$ and $\frac{d\Delta pK_1}{d\,(\text{cell volume})}$ must diminish as their denominators approach zero and the pro-

longations of the lines at low oxygen capacity and low pH will tend to converge and to approach the base line as an asymptote.

The chart is offered not as a final evaluation of the variations of ΔpK_1 and the determinants of these variations, but as the closest approximation as yet available. Such an approximation is essential to the proper interpretation of other data dealing with blood electrolyte equilibria. The relative accuracy of different portions of the chart is very variable. The portion indicated by solid lines is reasonably certain, the remainder is open to grave doubt, although it can be accepted as an expression of a general tendency. The material necessary to fill in the gaps in the chart can be accumulated only slowly, even in an active medical clinic. Recourse could be had to the artificial production of anemia and acidosis or alkalosis, either *in vitro* or *in vivo*; but this is not in keeping with the purpose of this work.

This raises the whole question of the propriety of drawing conclusions with regard to chemical and physiological constants from pathological blood. The answer to this is clear. Unless one turns to pathological blood one cannot study the effects of changes in the cell concentration or the pH of blood. Blood which has been depleted of its cells or rendered more acid by experimental methods, either *in vivo* or *in vitro*, is no more normal than the blood from a patient with severe primary or secondary anemia. Moreover, one of the necessary functions of the clinical chemist is to determine to what extent the results of normal control work can be applied to pathological material.

Something must be said about the method of constructing the correction chart. At first sight the method pursued by Warburg seems less empirical. Closer analysis shows that this is only an apparent distinction and Warburg has only obscured his empiricism by mathematics. The importance of Warburg's contribution to the clarification of the mathematical theory of the Henderson-Hasselbalch equation cannot be belittled; but his equations involve complicated variable functions, the variations of which can only be determined by experiment. Warburg's equation depends upon the proper estimation of the variation of the distribution of bicarbonate between cells and plasma. The uncertainty of this variation appears from Chart 2. The distribution coefficient is, in itself, a factor quite as complex as Δ pK_1 and its

variability in these experiments is quite as great. The correlation obtained by comparing ΔpK_1 directly with cell volume and pH is also far better than that obtained by Warburg's more devious method.

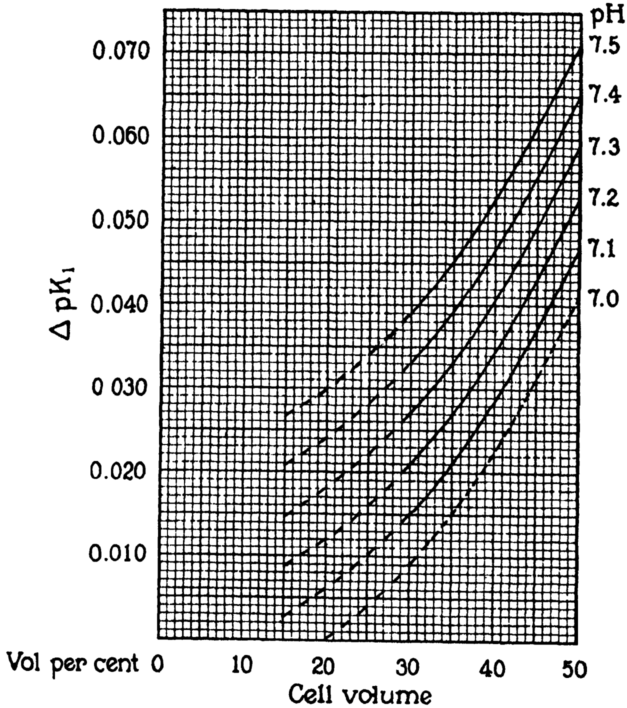

CHART 4. In Hasselbalch's equation (2) $[BHCO_3]$ = Total CO_2 − dissolved CO_2 and $[H_2CO_3]$ = dissolved CO_2.

Dissolved CO_2 may be derived by equation (9) or obtained from Chart 1, and subtracted from the total CO_2 found by analysis, to give $[BHCO_3]$. Log $[BHCO_3]$ − log (dissolved CO_2) is then determined and is added to 6.100, the value of pK_1 for plasma at 38°C. To the value thus obtained for pH is added the amount indicated in Chart 4 for the corresponding cell volume and pH.

For example, if log $[BHCO_3]$ − log (dissolved CO_2) = 1.200 and the cell volume of the blood is 40 volumes per cent, the pH uncorrected will be 6.100 + 1.200 = 7.300. The corrected factor for pH = 7.3 and cell volume = 40 is found from Chart 4 to be + 0.047. Therefore, the corrected pH will be 7.347.

TABLE III.

Case No.	CO_2 tension	O_2 capacity	Cell volume.	CO_2 content.		$[H_2CO_3]$		$[BHCO_3]$		pH = 6.100 + log $[BHCO_3]:[H_2CO_3]$		ΔpK_1 observed, Column 12 − Column 11.	ΔpK_1 calculated by Chart 4.	Difference, Column 14 − Column 13.
				Blood.	Plasma.	Blood.	Plasma.	Blood.	Plasma.	Blood.	Plasma.			
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
	mm. Hg	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*			
40	40	18.70	41.5	42.06	47.78	2.65	2.85	39.41	44.93	7.272	7.298	0.026	0.043	+0.017
42	40	17.15	36.5	46.10	53.76	2.68	2.85	43.42	50.91	7.310	7.352	0.042	0.038	−0.004
44	40	15.50	34.2	36.19	40.77	2.70	2.85	33.50	37.92	7.194	7.224	0.030	0.028	−0.002
45	40	18.15	41.1	49.45	58.60	2.65	2.85	46.80	55.75	7.347	7.391	0.044	0.047	+0.003
46	40	18.67	40.8	49.07	60.18	2.66	2.85	46.42	57.33	7.343	7.404	0.061	0.046	−0.015
47	40	18.45	41.1	45.51	52.70	2.65	2.85	42.86	49.85	7.309	7.343	0.034	0.045	+0.011
48	40	20.00	43.0	41.23	48.90	2.65	2.85	38.58	46.06	7.263	7.308	0.045	0.045	0.000
50	40	17.98	38.0	43.22	51.16	2.67	2.85	40.55	48.31	7.281	7.329	0.048	0.039	−0.009
51	40	18.01	44.3	42.23	51.13	2.64	2.85	39.59	48.28	7.276	7.329	0.053	0.048	−0.005
52	40	16.16	40.9	36.70	43.14	2.65	2.85	34.04	40.29	7.207	7.250	0.043	0.039	−0.004
53	40	17.24	36.2	53.60	63.30	2.68	2.85	50.92	60.45	7.379	7.427	0.048	0.042	−0.006
54	40	17.10	37.6	52.26	63.30	2.68	2.85	49.58	60.45	7.367	7.427	0.060	0.043	−0.017
55	40	19.08	39.6	45.90	54.56	2.66	2.85	43.24	51.71	7.311	7.359	0.048	0.043	−0.005
57	40	19.54	41.7	38.42	45.09	2.65	2.85	35.77	42.24	7.230	7.271	0.041	0.041	0.000
58	40	18.15	39.9	45.99	52.13	2.66	2.85	43.33	49.28	7.312	7.338	0.026	0.043	+0.017
59	40	20.60	43.7	44.41	55.13	2.65	2.85	41.76	52.28	7.299	7.363	0.064	0.049	−0.015
60	40	17.83	38.3	62.27	74.15	2.67	2.85	59.60	71.30	7.449	7.498	0.049	0.049	0.000
61	40	22.14	44.3	44.96	55.52	2.63	2.85	42.33	52.67	7.307	7.367	0.060	0.050	−0.010
63	40	16.06	35.9	38.18	43.40	2.67	2.85	35.51	40.55	7.224	7.253	0.029	0.032	+0.003
64	40	19.96	38.6	48.00	57.46	2.67	2.85	45.33	54.61	7.330	7.382	0.052	0.042	−0.010
65	40	20.40	41.5	49.83	59.33	2.65	2.85	47.18	56.48	7.351	7.397	0.046	0.048	+0.002

66	40	17.50	36.6	47.00	56.40	2.68	2.85	44.32	53.55	7.318	7.374	0.056	0.038	−0.018
67	40	17.60	36.7	53.70	63.50	2.68	2.85	51.02	60.65	7.380	7.428	0.048	0.042	−0.006
68	40	13 90	30.8	47.20	52.50	2.70	2.85	44.50	49.65	7.317	7.341	0.024	0.030	+0.006
69	40	20.20	43.4	49.30	60.10	2.65	2.85	46.65	57.25	7.346	7.403	0.057	0.051	−0.006
71	40	19.72	38.3	31.47	34.22	2.67	2.85	28.80	31.37	7.133	7.142	0.009	0.030	+0.021
72	40	19.90	43.3	47.70	57.00	2.65	2.85	45.05	54.15	7.330	7.379	0.049	0.050	+0.001
74	40	18.40	39.9	47.83	56.02	2.66	2.85	45.17	53.17	7.330	7.371	0.041	0.045	+0.004
76	40	22.35	47.2	41.75	51.10	2.63	2.85	39.13	48.25	7.273	7.329	0.056	0.053	−0.003
77	40	21.30	45.1	60.80	76.50	2.64	2.85	58.17	73.65	7.444	7.512	0.068	0.060	−0.008
79	40	20.13	46.2	42.85	50.62	2.63	2.85	40.22	47.77	7.285	7.324	0.039	0.052	+0.013
80	40	22.25	46.8	43.95	52.10	2 63	2.85	41.32	49.25	7.297	7.338	0.041	0.056	+0.015
83	40	18.80	40.0	49.10	57.40	2.66	2.85	46.44	54.55	7.342	7.383	0.041	0.045	+0.004
86	40	20.90	41.9	45.40	53.35	2.63	2.85	42.77	50.50	7.311	7.348	0.037	0.047	+0.010
27	40	12.60	29.5	45.10	49.85	2.72	2.85	42.38	47.00	7.293	7.317	0.024	0.026	+0.002
30	40	9.90	21.4	29.30	30.75	2.75	2.85	26.55	27.90	7.085	7.091	0.006	0.006	0.000
34	40	11.58	29.6	44.70	48.75	2.72	2.85	41.98	45.90	7.288	7.307	0.019	0.026	+0.007
70	40	7.58	15.6	40.00	41.46	2.78	2.85	37.22	38.61	7.227	7.232	0.005	0.010	+0.005
73	40	7.76	17.6	31.50	32.10	2.77	2.85	28.73	29.25	7.116	7.111	−0.005	0.004	+0.009
75	40	14.90	28.9	47.40	52.25	2.72	2.85	44.68	49.40	7.316	7.339	0.023	0.027	+0.004
81	40	10.95	25.4	28.14	29.59	2.73	2.85	25.41	26.74	7.069	7.072	0.003	0.008	+0.005
82	40	6.39	14.7	30.95	32.40	2.78	2.85	28.17	29.55	7.106	7.116	0.010	0.002	−0.008
85	40	7.21	15.7	31.47	32.70	2.78	2.85	28.69	29.85	7.113	7.120	0.007	0.003	−0.004
88	40	10.88	23.0	40.90	42.05	2.75	2.85	38.05	39.20	7.241	7.238	−0.003	0.017	+0.020
28	30	18.58	40.9	40.80	48.80	1.99	2.14	38.81	46.66	7.390	7.439	0.049	0.050	+0.001
	60	18.58	41.8	53.35	62.80	3.98	4.27	49.37	58.53	7.194	7.237	0.043	0.039	−0.004
32	30	16.20	38.5	24.85	28.45	2.00	2.14	22.85	26.31	7.158	7.190	0.032	0.032	0.000
	60	16.20	38.7	35.65	40.05	4.01	4.27	31.64	35.78	6.997	7.023	0.026	0.029	+0.003

TABLE III—*Concluded.*

Case No.	CO_2 tension.	O_2 capacity.	Cell volume.	CO_2 content.		$[H_2CO_3]$		$[BHCO_3]$		pH = 6.100 + log $[BHCO_3]:[H_2CO_3]$		ΔpK_1 observed, Column 12—Column 11.	ΔpK_1 calculated by Chart 4.	Difference, Column 14—Column 13.
				Blood.	Plasma.	Blood.	Plasma	Blood.	Plasma.	Blood.	Plasma			
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
	mm. Hg	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*			
33	30	22.40	46.6	39.23	47.30	1.98	2.14	37.25	45.16	7.374	7.424	0.050	0.058	+0.008
	60	22.40	47.6	52.80	63.60	3.95	4.27	48.85	59.33	7.192	7.243	0.051	0.048	−0.003
35	30	17.86	35.2	43.27	51.30	2.02	2.14	41.25	49.16	7.410	7.461	0.051	0.041	−0.010
	60	17.86	36.2	55.37	64.88	4.03	4.27	51.34	60.61	7.205	7.252	0.047	0.032	−0.015
38	30	16.10	33.6	35.68	38.38	2.02	2.14	33.66	36.24	7.322	7.329	0.007	0.034	+0.027
	60	16.10	34.2	46.15	50.50	4.04	4.27	42.11	46.23	7.118	7.134	0.016	0.023	+0.007
43	30	21.30	45.7	38.01	46.40	1.98	2.14	36.03	44.26	7.360	7.416	0.056	0.056	0.000
	60	21.30	46.7	51.66	61 73	3.95	4.27	47.71	57.46	7.182	7.229	0.047	0.046	−0.001
49	30	16.49	35.8	42 38	47.80	2.01	2.14	40.37	45.66	7.403	7.429	0.026	0.043	+0.017
	60	16.49	38.0	53.62	61 46	4.02	4.27	49 60	57.19	7.191	7.227	0.035	0.033	−0.003
56	30	18.90	43.3	46.81	57.79	1.99	2.14	44 82	55.65	7.453	7.515	0.062	0.057	−0.005
	60	18.90	43.1	61.08	73 39	3.98	4.27	57.10	69.12	7 257	7.309	0.052	0.045	−0.007
62	30	21.31	45.4	39.69	48.66	1.97	2.14	37.72	46 52	7.382	7.437	0.055	0.056	+0.001
	60	21.31	46.1	53.26	63.98	3.94	4.27	49.32	59.71	7.198	7.246	0.048	0.046	−0.002
84	30	17.76	38.9	42.40	50.20	2.00	2.14	40.40	48.06	7.405	7 451	0.046	0.047	+0.001
	60	17.76	39.6	54.85	64.10	4.00	4.27	50.85	59.83	7.204	7.246	0.042	0.037	−0.005
87	30	21.30	43.3	47.60	57.90	1.98	2.14	45 62	55.76	7.462	7.516	0.054	0.058	+0.004
	60	21.30	44.0	61.75	73.60	3.97	4.27	57.48	69.33	7.261	7.310	0.049	0.047	−0.002
29	30	12.38	30.3	23.75	25.70	2.03	2.14	21.72	23.56	7.129	7.142	0.013	0.018	+0.005
	60	12.38	29.3	33.90	36.50	4.07	4.27	29.83	32 23	6 965	6.978	0.013	0.013	0.000

Throughout this discussion nothing has been said of the actual value of pK_1. The value of 6.100 for the pK_1 of plasma has been accepted as a constant. How far such an assumption is justified can only be determined by electrometric methods.

CONCLUSIONS.

The CO_2 content of both blood and plasma and the oxygen capacity and cell volume of blood exposed to known mixtures of air and CO_2 have been determined.

These data support the contention of Warburg that pK_1 of the Henderson-Hasselbalch equation shows an apparent variation with hemoglobin concentration and with pH.

Warburg's correction factors have, however, proved unsatisfactory when applied to the data of these experiments.

A quantitative estimation of the variations of pK_1 has been made and a series of curves has been presented by means of which corrections can be made.

By means of these curves the pH of plasma can be predicted from the CO_2 content and CO_2 tension of whole blood with a mean error of less than $\pm$ 0.01 pH.

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STUDIES OF THE CARBON DIOXIDE ABSORPTION CURVE OF HUMAN BLOOD.

II. THE NATURE OF THE CURVE REPRESENTING THE RELATION OF pH TO $BHCO_3$.

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Warburg (1922) and Van Slyke, Austin, and Cullen (1922) have recently shown independently that if the carbon dioxide absorption curve of blood or plasma is drawn with pH plotted against bicarbonate instead of the ordinary way, it approximates a straight line. Although they recognize that this relation rests on an empirical basis only, they have, in some instances, corrected experimental data to conform to the theory that the curve so drawn is a straight line.

There is no doubt that most of the absorption curves available in the literature could be drawn as straight lines without altering the observed values beyond the limits of experimental error in the methods by which the latter were obtained. But it is possible that such a relation is only an apparent one and that, as methods become more refined it will prove only approximate. It is possible that it exists only within certain limits of pH or CO_2 tension. The importance of determining the limits within which one can assume the straight line relation, and the error involved in such an assumption, is evident. If the approximation were sufficiently close one could construct an absorption curve, or a certain portion of an absorption curve from two points, with great economy of time and material.

EXPERIMENTAL.

Partly with the purpose of investigating the accuracy of this relation we have determined the carbon dioxide capacity of the blood of twenty-six subjects at 20, 40, and 80 mm. CO_2 tension, at 38°C. by methods similar to those outlined in the preceding paper. In order to render complete saturation and equilibrium more certain, however, only 8 to 10 cc. of blood were saturated in each tonometer. The results of the experiments are presented in Table I. The corrections for dissolved CO_2 and for pK_1 presented in the preceding paper were employed in the calculation of bicarbonate and pH values.

From the values obtained for bicarbonate and pH at the three points the slopes of the curves between each two points were calculated by means of the equation

$$\frac{d\,[BHCO_3]}{dpH} = m \tag{1}$$

where m is the slope. The mathematical average of the three slopes thus obtained was substituted in the straight line equation

$$[BHCO_3] = m\,pH + b \tag{2}$$

and a value of b at each point was calculated. The three values of b were averaged. By means of the average values of m and b thus calculated a straight line curve was constructed and the deviation of each point from this line was calculated by equation (2), using observed pH values to estimate $[BHCO_3]$. The lines were then adjusted mathematically to give the least possible deviation of all three points. The deviations thus obtained are given in the last three columns of Table I.

DISCUSSION.

The deviations from the straight line equation found in these experiments are too large to be ascribed to errors in the experimental methods employed unless the latter have been underestimated. Control experiments aimed to test the accuracy of the methods have been uniformly satisfactory. The relations of $[BHCO_3]$ to pH are defined by the familiar Henderson-Hasselbalch equation

$$pH = pK_1 + \log \frac{[BHCO_3]}{[H_2CO_3]} \tag{3}$$

If there were anything in this equation to suggest that the relation of pH to [$BHCO_3$] was linear the evidence of the control work would have to be discounted. But the Henderson-Hasselbalch equation neither suggests nor precludes such a linear relation. The results of our experiments are, furthermore, quite in keeping with those of other observers, who have employed similar methods on comparable material (Van Slyke, Austin, and Cullen, 1922; and Doisy, Eaton, and Chouke, 1922). Under these conditions it does not seem proper to consider the straight line equation as more than an approximation.

The fact that the straight line relation does not hold in these and similar experiments does not preclude the possibility that such a relation really exists. In this analysis it was assumed that the Henderson-Hasselbalch equation was correct and that the exact values of pK_1 and pH were available. The assumption of the essential validity of the equation is probably permissible; but the accuracy of the values ascribed to pH and pK_1 in whole blood is open to serious question. In the preceding paper of this series it was shown that, by using the proper corrections for pK_1 in whole blood the pH of plasma could be predicted with a mean error of only 0.007 pH. In some blood samples, however, a much larger error was found, in one instance as much as 0.03 pH. The divergence of these curves from the straight line form may be due to the assumption of improper pK_1 values for the calculation of pH.

The limits 20 to 80 mm. were chosen with a definite point in view. Most normal and pathological cases will be included within these tensions. The experiments further include bloods varying in hemoglobin concentration from an O_2 capacity of 2.65 volumes per cent to one of 21.6, and in pH (at 40 mm.) from 7.117 to 7.376. It should be possible from these data to estimate with some accuracy the degree of error involved in assuming the straight line relation and drawing the absorption curve from two points. The average deviation of the middle point from the straight line connecting the two end-points is $\pm$ 1.26 volumes per cent of [$BHCO_3$]. The average change of pH between 20 and 80 mm. is 0.206. The majority of the curves assume a shape convex to the abscissa, but curves are found with the opposite inflection. Doisy, Eaton, and Chouke (1922) have encountered similar differences of inflection.

TABLE I.

Experiment No.	O_2 capacity.	Cell volume.	Total CO_2. 20 mm. CO_2.	Total CO_2. 40 mm. CO_2.	Total CO_2. 80 mm. CO_2.	[$BHCO_3$] 20 mm. CO_2.	[$BHCO_3$] 40 mm. CO_2.	[$BHCO_3$] 80 mm. CO_2.	pH 20 mm CO_2	pH 40 mm. CO_2	pH 80 mm. CO_2.	Slope, *m*. 20 to 40 mm CO_2.	Slope, *m*. 40 to 80 mm. CO_2	Average *m*.	Average *b*.	[$BHCO_3$] calculated, at 20 mm.	[$BHCO_3$] calculated, at 40 mm.	[$BHCO_3$] calculated, at 80 mm.	Difference between observed and calculated values of [$BHCO_3$] at 20 mm.	Difference between observed and calculated values of [$BHCO_3$] at 40 mm.	Difference between observed and calculated values of [$BHCO_3$] at 80 mm.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol per cent*	*vol. per cent*	*vol. per cent*								*vol. per cent*	*vol. per cent*	*vol. per cent*			
1	10.13	26.0	33.15	41.80	53.75	31.79	39.07	48.29	7.502	7.276	7.055	−32.2	−41.7	−36.9	308 3	31.32	39.82	48.02	−0.47	+0.75	−0.27
2	10.83	26.0	39.35	48.45	60.50	36.99	45.72	55.04	7.572	7.348	7.114	−39.0	−39.8	−39.4	335.5	37.15	45.65	55.25	+0.16	−0.07	+0.21
3	21.33	48.5	33.75	46.40	62.25	32.44	43.77	57.00	7.562	7.379	7.182	−61.9	−67.1	−64.5	520.3	32.34	44.14	56.74	−0.10	+0.37	−0.26
4	18.83	41.5	35.10	44.40	58.90	33.77	41.74	53.58	7.563	7.341	7.137	−35.9	−58.0	−46.8	387.0	32.83	43.23	53.03	−0.94	+1.49	−0.55
5	18.40	41.0	33.35	42.70	58.50	32.02	40.04	53.18	7.538	7.327	7.133	−38.0	−67.7	−52.6	427.6	30.93	41.83	52.43	−1.09	+1.79	−0.75
6	15.08	29.0	26.60	36.35	47.25	25.25	33.64	41.82	7.402	7.214	6.996	−44.6	−37.5	−40.9	328.2	25.34	33.24	42.14	+0.09	−0.40	+0.32
7	18.45	43.5	34.40	47.65	58.25	33.08	45.00	52.95	7.560	7.380	7.136	−66.2	−32.6	−48.6	401.4	33.88	42.58	54.58	+0.80	−2.42	+1.63
8	10.70	21.5	24.60	33.50	43.80	23.23	30.75	38.30	7.350	7.158	6.943	−39.2	−35.1	−37.1	296.2	23 39	30.49	38.39	+0.16	−0.26	+0.09
9	21.60	44.5	28.65	39.10	53.15	27.33	36.46	47.88	7.474	7.287	7.093	−48.8	−58.8	−53.9	429.9	27.02	36.92	47.72	−0.31	+0.46	−0.16
11	19.10	42.0	27.95	39.20	53.20	26.62	36.54	47.89	7.453	7.280	7.086	−57.3	−58.5	−57.9	458.3	26.58	36.58	47.88	−0.04	+0.04	−0.01
12	4.23	13.0	45.20	52.00	60.35	43.81	49.21	54.77	7.629	7.362	7.092	−20.2	−20 6	−20.4	199.5	43.76	49.26	54.76	−0.05	+0.05	−0.01
14	11.57	29.0	37.70	47.40	60.70	36.35	44.69	55.27	7.570	7.344	7.123	−36.9	−47.9	−42.3	356.3	36.07	45.47	54.77	−0.28	+0.78	−0.50
15	18.66	44.5	34.60	45.00	60.50	33.28	42.36	55.22	7.564	7.355	7.158	−43.4	−65.3	−54.2	442.7	32.35	43.85	54.65	−0.93	+1.49	−0.57
16	19.93	45.5	30.35	41.15	56.80	29.03	38.51	51.52	7.502	7.313	7.128	−50.2	−70.3	−60.2	480.1	28.29	39.89	50.89	−0.74	+1.38	−0.63
17	9.73	23.5	22.40	30.65	41.35	21.03	27.91	35.86	7.305	7.117	6.915	−36.6	−39.4	−38.0	298.6	20.80	28.20	35.80	−0.23	+0.29	−0.06
18	8.58	19.5	30.50	37.05	47.40	29.12	34.29	41.88	7.448	7.204	6.980	−21.2	−33.9	−27.5	233.5	28.56	35.26	41.46	−0.56	+0.95	−0.42
19	8.36	21.5	29.85	37.95	47.90	28.48	35.20	42.40	7.444	7.220	6.987	−30.0	−30.9	−30.5	255.5	28.36	35.36	42.36	−0.12	+0.16	−0.06

20	19.90	45.5	28.95	39.55	54.15	27.63	36.91	48.87	7.480	7.294	7.103	−49.9	−62.6	−56.3	448.5	27.34	37.74	48.34	−0.29	+0.83	−0.53
21	20.98	52.5	23.50	33.35	49.35	22.20	30.75	44.14	7.398	7.227	7.073	−50.0	−86.9	−68.1	525.0	20.76	32.36	42.96	−1.00	+1.95	−0.84
22	21.40	50.0	29.25	39.70	55.55	27.95	37.09	50.32	7.499	7.308	7.128	−47.8	−73.5	−60.5	480.9	27.02	38.72	49.62	−0.93	+1.63	−0.70
23	2.65	5.4	35.95	41.75	49.10	34.54	38.93	43.45	7.489	7.240	6.986	−17.6	−17.8	−17.7	167.1	34.54	38.94	43.44	0.00	+0.01	−0.01
24	10.40	23.0	37.90	46.25	58.55	36.53	43.51	53.06	7.560	7.321	7.092	−29.2	−41.7	−35.4	303.7	35.90	44.60	52.60	−0.63	+1.09	−0.46
25	17.10	38.0	26.80	36.00	48.75	25.47	33.34	43.40	7.426	7.232	7.032	−40.6	−50.3	−45.5	363.1	25.10	34.00	43.10	−0.37	+0.66	−0.30
26	4.65	12 0	26.40	33.50	41.63	25.01	30.74	36.08	7.371	7.151	6.913	−26.0	−22.4	−24.2	203.6	25.18	30.38	36.28	+0.19	−0.36	+0.20
27	12.60	29.5	33.35	45.10	55.55	32.00	42.31	49.97	7.512	7.306	7.064	−50.0	−31.7	−40.6	337.7	32.69	40.89	50.69	+0.69	−1.42	+0.72
31	19.40	43.5	32.55	45.20	59.30	31.23	42.55	54.00	7.533	7.355	7.145	−63.6	−54.5	−58.9	475.4	31.46	42.06	54.26	+0.23	−0.49	+0 26

If the logarithm of CO_2 tension, or log $[H_2CO_3]$, is plotted against the logarithm of the CO_2 content of the blood, or log $[BHCO_3]$, the resulting curve approximates the straight line form just as closely as does the curve of pH against $[BHCO_3]$. In fact, in the

TABLE II.

Experiment No.	CO_2 at			log CO_2 at			Slope m 20 to 80 mm.	CO_2 calculated at 40 mm.	Difference between observed and calculated CO_2 at 40 mm.
	20 mm.	40 mm.	80 mm.	20 mm.	40 mm.	80 mm.			
	vol. per cent	*vol. per cent*	*vol. per cent*					*vol. per cent*	
1	33.15	41.80	53.75	1.5205	1.6212	1.7304	0.3486	41.79	−0.01
2	39.35	48.45	60.50	1.5949	1.6853	1.7818	0.3100	48.79	+0.34
3	33.75	46.40	62.25	1.5283	1.6665	1.7941	0.4413	45.62	−0.78
4	35.10	44.40	58.90	1.5453	1.6474	1.7701	0.3730	45.46	+1.06
5	33.35	42.70	58.50	1.5231	1.6304	1.7672	0.4050	44.11	+1.41
6	26.60	36.35	47.25	1.4249	1.5605	1.6744	0.4145	35.44	−0.91
7	34.40	47.65	58.25	1.5366	1.6781	1.7653	0.3800	44.78	−2.87
8	24.60	33.50	43.80	1.3909	1.5250	1.6415	0.4160	32.80	−0.70
9	28.65	39.10	53.15	1.4571	1.5922	1.7255	0.4460	39.01	−0.09
11	27.95	39.20	53.20	1.4464	1.5933	1.7259	0.4640	38.57	−0.63
12	45.20	52.00	60.35	1.6551	1.7160	1.7807	0.2085	52.22	+0.22
14	37.70	47.40	60.70	1.5763	1.6758	1.7832	0.3435	47.83	+0.43
15	34.60	45.00	60.50	1.5391	1.6532	1.7818	0.4030	45.77	+0.77
16	30.35	41.15	56.80	1.4822	1.6144	1.7544	0.4525	41.53	+0.38
17	22.40	30.65	41.35	1.3503	1.4864	1.6165	0.4420	30.41	−0.24
18	30.50	37.05	47.40	1.4843	1.5688	1.6758	0.3180	38.03	+0.98
19	29.85	37.95	47.90	1.4749	1.5792	1.6803	0.3410	37.79	−0.16
20	28.95	39.55	54.15	1.4617	1.5972	1.7336	0.4490	39.52	−0.03
21	23.50	33.35	49.35	1.3711	1.5231	1.6933	0.5355	34.05	+0.70
22	29.25	39.70	55.55	1.4661	1.5988	1.7447	0.4630	40.32	+0.62
23	35.95	41.75	49.10	1.5557	1.6207	1.6911	0.2250	42.01	+0.26
24	37.90	46.25	58.55	1.5786	1.6651	1.7675	0.3140	47.13	+0.88
25	26.80	36.00	48.75	1.4281	1.5563	1.6880	0.4320	36.17	+0.17
26	26.40	33.50	41.63	1.4216	1.5250	1.6194	0.3290	33.17	−0.33
27	33.35	45.10	55.55	1.5231	1.6542	1.7447	0.3680	43.05	−2.05
31	32.55	45.20	59.30	1.5126	1.6551	1.7731	0.4330	43.92	−1.28

experiments of this series the correlation is somewhat better. In the last column of Table II is shown the deviation of the observed CO_2 content at 40 mm. from the straight line connecting the 20 and 80 mm. points, plotted logarithmically as CO_2 tension against

CO_2 content. The average deviation is $\pm$ 0.70 volume per cent as against an average deviation of $\pm$ 1.26 obtained when [$BHCO_3$] was plotted against pH. The two methods have also been applied to the five oxygenated blood curves presented by Van Slyke, Hastings, Heidelberger, and Neill (1922) with equally satisfactory results.

Barcroft, Bock, Hill, Parsons, Parsons, and Shoji (1922) have suggested that the absorption curve approaches a straight line when plotted as C_H against CO_2. We have arranged our data in this way, and find that the divergence from the straight line is greater than that obtained by either of the methods discussed above.

CONCLUSIONS.

A series of twenty-six CO_2 absorption curves between the limits of 20 and 80 mm. has been determined from the blood of twenty-six normal and pathological subjects. The bloods studied varied in O_2 capacity from 2.65 to 21.6 volumes per cent and in pH (at 40 mm.) from 7.117 to 7.376.

From the data thus obtained values for pH were calculated by means of the Henderson-Hasselbalch equation with the correction factors presented in the preceding paper of this series.

When these pH values were plotted against [$BHCO_3$] the resulting curves obtained were found to deviate from the straight line form assumed by Van Slyke, Austin, and Cullen (1922) and Warburg (1922). These deviations were greater than the estimated errors in the experimental methods employed.

The average deviation of the 40 mm. point from the straight line connecting the 20 and 80 mm. points was $\pm$ 1.26 volumes per cent. The average change in pH between 20 and 80 mm. was 0.206.

The curve obtained by plotting the CO_2 content against CO_2 tension expressed in logarithmic form also approximates a straight line. This method gives somewhat better correlation in this series of experiments than that obtained by plotting pH against [$BHCO_3$]. The average deviation of the 40 mm. point from the line connecting the 20 and 80 mm. points was $\pm$ 0.70 volume per cent.

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THE UTILIZATION OF CARBOHYDRATE BY RATS DEPRIVED OF VITAMINE B.*

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The function of vitamine B in the animal organism is still a subject of speculation and surmise. Many of the suggested explanations of its action have been found on further examination to be partial, or have been definitely negated by new evidence; the vital processes in which vitamine B is not concerned can, therefore, be more explicitly stated than can those in which it plays a part. This paper presents some data on the suggested relation of vitamine B to the utilization of sugar.

A connection of vitamine B with the metabolism of carbohydrate was first suggested by Funk (1) in 1914 on the basis of experiments with pigeons in which polyneuritis developed more rapidly with increasing quantities of rice fed. Subsequent investigators in part confirmed, in part rejected, the idea. Similar experiments on rats (2) were not convincing.

That some of the processes of metabolism are disturbed by lack of vitamine B is obvious and since the course of protein seems in no way altered either in the rat (3) or in the dog (4), the processes involved when vitamine B is absent from the diet in mineral metabolism and in energy metabolism are next to be examined. That the utilization of Ca, Mg, and P in the human organism is apparently improved by the addition of yeast to a basal diet (5) is in itself a striking fact and indicates the importance of further work in this direction.

A derangement of some phase of energy metabolism is to be inferred from the subnormal body temperature noted by many observers and in the search for an explanation the question arises

*A report of the results in this paper was presented at the Toronto meeting of the American Society of Biological Chemists (Mattill, H. A., *J. Biol. Chem.*, 1923, lv, p. xxv).

whether the oxidizing power of the tissues has been impaired, whether their oxygen supply is subnormal, or whether oxidizable material is being withheld from them. This last possibility is supported by the loss of appetite and the alimentary stasis which is especially evident in birds. The immediate effect of vitamine B administration in restoring appetite and motility points in the same direction although the mechanism of this stimulation is not understood. That the tissues lack oxygen might be inferred from the decreased Hb and red cell count in polyneuritic pigeons (6, 7), but a lack of vitamine does not cause anemia in rats (8). Rather more suggestive is the idea that the tissues are being deprived of some agency which facilitates oxidation. A lowered tissue catalase in avian polyneuritis with restoration to normal by administration of vitamine B was recorded by Dutcher (9). However, a low catalase content of the tissues of fasting birds (10) and dogs (11) has also been observed. Findlay (12) indicated a reduction of the liver glyoxalase in beri-beri pigeons and a definite increase following vitamine B administration. Of interest also is the statement of Abderhalden and Wertheimer (13) who conclude from observations on beri-beri pigeons and their tissues that vitamines (they do not specify but they used yeast) accelerate respiratory processes. A study of the charts, however, fails to convince. They also show (14) that normal fasting pigeons are less sensitive to decreased oxygen (barometric) pressure than polyneuritic birds. Hess (15), studying the oxidizing capacity of tissues by their ability to reduce *m*-nitrobenzene, concluded that avitaminosis is a depletion of the respiratory enzymes of the tissue cells, reproducible by mild HCN poisoning, an analogy which Abderhalden and Wertheimer (14) enthusiastically destroy.

There are numerous observations showing a reduction of gaseous metabolism in the absence of vitamine B. Most recent and complete are those of Anderson and Kulp (16) who cite the previous literature. From their results on poultry which were in substantial agreement with the findings of previous investigators on pigeons it appears that heat production declined with advancing vitamine B starvation and finally reached levels which were 40 to 50 per cent below the normal basal. This was coincident with decreased food consumption, but was dependent on other

factors since forcibly fed birds utilized such food very slowly. There was, however, no noticeable change in the respiratory quotient. Until a short time before polyneuritis developed quotients approaching or exceeding unity were observed during 3 to 4 hours following rice feeding. After symptoms of paralysis appeared, although the crop contained undigested rice, the quotient seldom rose above 0.75, a quotient not much below their normal basal (18 hours) and one which is obtained from most animals during the early stages of fasting. The evidence is almost complete, therefore, that the lowered heat production and the low quotients in polyneuritic birds are not the results of any inability on the part of the tissues to burn sugar (or other materials), but indicate that the organism is in a fasting condition due to a failure of the functions of the alimentary tract. An objection to this interpretation might be found in the immediately increased heat production on administering yeast or its alcoholic extract (17); since such treatment immediately restores motility to the alimentary tract[1] and stimulates absorption (18) the increased tissue oxidation might well come from a renewed food supply. A more serious objection is that implied in the observations of Abderhalden and his coworkers (19) that the gaseous metabolism of isolated organs and tissues of polyneuritic pigeons is much less intense than that of similar structures from normal birds, and is raised toward normal values when yeast extracts are introduced into the surrounding liquid; also that such extracts do not raise the oxidation level of normal organs. He also finds (20), however, that a great many substances which are not vitamines increase the oxygen consumption of blood and tissues. A further fact tending to support a reduced oxidation capacity of tissue as a cause of low heat production is the observation of Funk and von Schönborn (21), showing high blood sugar and low liver glycogen in polyneuritic birds as compared with normals, and the approach to normal values when vitamine B was administered. Repetition and amplification of this work (22) did not provide additional support for this conclusion since both liver glycogen and blood

[1] In unpublished experiments on chickens I frequently verified the common observation that the fully gorged crop of a bird without vitamine B is rapidly emptied when small amounts (2 to 3 cc.) of autolyzed yeast are administered.

sugar were lower in rice-fed pigeons receiving 10 gm. of glucose daily than in similar rice-fed pigeons without glucose addition. Also the liver glycogen of rice-fed pigeons was greater than that of normal pigeons when both sets of animals were given glucose daily as well as when this was omitted. The determination of blood sugar in small animals is fraught with great uncertainty whether an anesthetic is employed or not, and while acetone has recently been reported in the expired air of rice-fed pigeons (14) they do not show a lowered alkali reserve (7). Polyneuritic fowls, on the other hand, show a lowered alkali reserve according to Japanese investigators (23). An increase in the blood fat of beri-beri pigeons as well as in pigeons subjected to brief fasting (3 days) has been indicated (24). In human beri-beri the fat content of the blood is reported as far below normal (25), and in beri-beri infants (26) blood sugar is said to be subnormal.

Whatever facts may be urged in favor of the view that polyneuritis involves an impairment of the oxidative processes in the tissues, especially as regards carbohydrate, the one fact remains as demonstrated by Anderson and Kulp (16) that poultry well advanced toward polyneuritis still show high respiratory quotients after rice feeding. This conclusion is supported by the observations of Gerstenberger and Burhans (27) to the effect that polyneuritic pigeons can burn carbohydrate completely. The details of their respiration experiments have not yet appeared; their animals may have been more or less "polyneuritic" than Anderson's but unless the processes of carbohydrate combustion suddenly fail in the terminal stages, and perhaps they do, their gradual impairment, which is the point at issue, remains unproved.

The only respiration experiments on rats deprived of vitamine B appear to be those of Gulick (28) who compared the CO_2 output of three male litter mates, one normally fed, a second deprived of water-soluble vitamine, and a third fed adequate food but in no greater amounts than were consumed by the animal on the defective diet. Both of the underfed rats were stunted and showed about one-third of the basal gas exchange of the normally fed rat; since their calorie intake was similar and their vitamine intake different the deficient calorie intake was held chiefly responsible for the lowered metabolism. It seemed desirable to have further information on the course of respiratory metabolism in

vitamine B-deprived rats, especially with reference to their respiratory quotients.

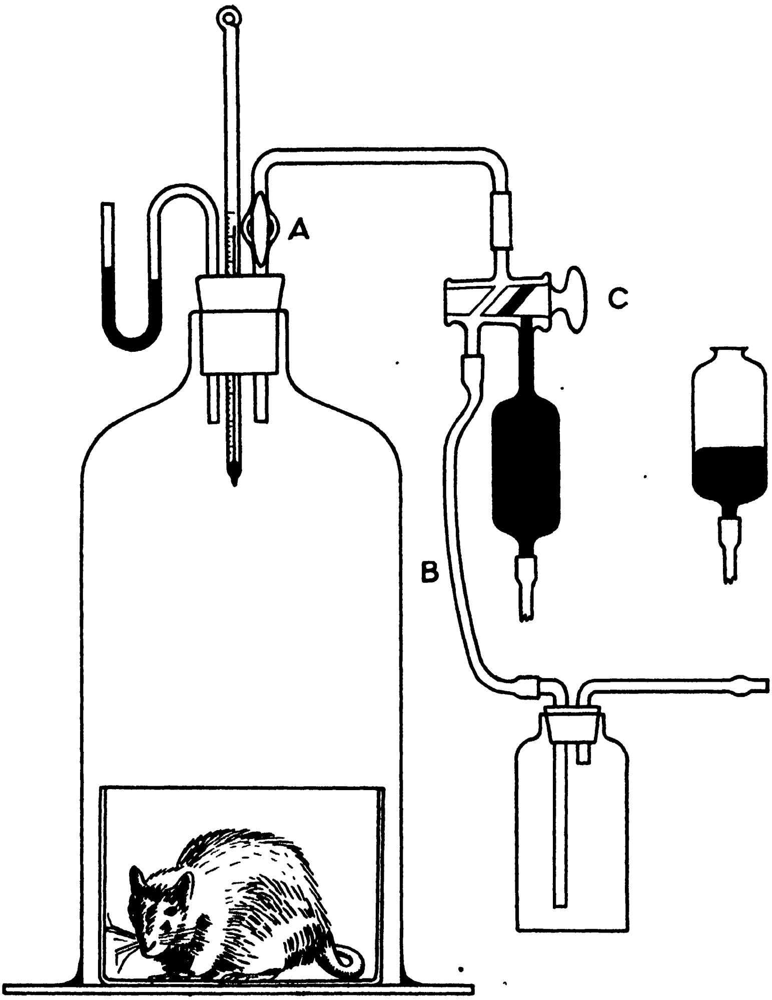

Fig. 1.

The respiration apparatus used in this work (Fig. 1) consists of a bell jar of 5 to 6 liters capacity which rests on a vaselined glass plate. A crystallizing dish slightly smaller in diameter than

the bell jar holds the animal and keeps it from contact with the greased plate. A 3-hole stopper in the opening at the top of the bell jar carries a thermometer, a small mercury manometer, and a glass tube for withdrawing the air sample. This tube has a stop-cock just above the stopper and is connected to the usual form of gas sampling tube containing mercury. During an interval of 10 to 20 minutes a full grown rat under such a jar produces sufficient changes (0.75 to 2 per cent) in the oxygen and carbon dioxide content of the confined air, that is, changes determinable with sufficient accuracy when a sample is analyzed in the Haldane apparatus, to serve as a basis for the calculation of respiratory quotients. When a period is about to be completed the manometer is read after gentle tapping, stop-cock *A* is opened and a small amount of the contained air is first withdrawn through the side tube *B*, in order to wash out the connecting tube. During this withdrawal valve *C* is turned so as to connect the sampling tube with the jar. Alternate raising and lowering of the mercury reservoir eight to ten times so that mercury almost refills the sampling tube each time serves to stir the air in the bell jar sufficiently to assure a uniform sample. After each determination the bell jar is removed from the plate and placed over an opening to the outside air with the stopper removed. An alcohol flame under the bell jar provides sufficient gaseous change for an alcohol check in from 2 to 5 minutes, depending on the size of the flame. With the exact volume of the bell jar known as well as the temperature and barometric pressure (corrected by the manometer on the bell jar which usually shows a 5 to 6 mm. rise due to temperature change inside), the data are at hand for the calculation of the total heat production. These data have proved extremely variable because of the inevitable movements of the animals and the short period of observation. Inasmuch as data on respiratory quotient were primarily desired the apparatus was not modified; it could easily be modified to give satisfactory data on total heat production by providing another opening low on the side of the bell jar to allow for a circulation of outside air. After the animal quieted down this circulation could be stopped and the stationary air could be sampled in the usual manner 10 to 20 minutes later. Possibly even with this arrangement properly worked out the data on total heat production would not be as accurate as those obtained in

a closed or open circuit apparatus. Certainly the data on rats reported by Asher and Danoff (29) using the Haldane (30) open circuit apparatus are strikingly consistent and leave nothing to be desired in the way of accuracy. They are almost too accurate. With the Haldane open circuit type a single determination requires at least 3 to 4 hours as against the possibility of determining the respiratory quotient of two or three animals in 1 hour with the apparatus just described. Changes in respiratory quotient due to food ingestion can also be much more closely followed for this reason; and because of its simplicity the bell jar arrangement with the Haldane gas analysis apparatus should be useful in laboratory instruction in nutrition where long periods of observation are not usually possible.

The animals used in these experiments were fed a synthetic ration containing casein 18, starch 50, lard 23, salts 4, and butter fat 5 per cent. The casein was freed from vitamine B by the dialysis method of McCollum. Control animals received this ration with 5 per cent of dry baker's yeast in place of an equal weight of starch. No record was kept of the food consumption. The animals were weighed frequently in order that a fair constancy of weight on the complete ration and the percentage loss in weight on the incomplete ration might be ascertained. Determinations of respiratory quotients were made on the vitamine-deprived animals at various times during the progress of their loss in weight, in both fasting and non-fasting conditions and after cane-sugar and glucose administration, and also on the control animals under similar conditions. The results are given in Tables I and II.[2] With few exceptions the basal respiratory quotients of animals on adequate food are seen to vary between 0.73 and 0.78, the average of thirty determinations being 0.755. Basal quotients of animals deprived of vitamine B vary from 0.70 to 0.78 and the average of fifteen determinations on these animals is 0.745. Vitamine B-deprived animals thus show the same basal quotients as those upon adequate food. Non-fasting quotients show a wider variation in both sets of animals; those on adequate food

[2] Due to unsuspected leaks in the respiration apparatus or in the Haldane-Henderson machine occasionally several determinations or even a whole series of observations had to be discarded because calculations were deferred until all the analyses should be completed.

vary from 0.79 to 0.97, with an average of 0.84 (twenty-three determinations); those on an inadequate ration range from 0.71 to 0.87 with an average of 0.78 in nineteen determinations. This difference is a reflection of the semifasting condition of vitamine-deprived animals.

When normal animals in a basal condition ate cane-sugar the respiratory quotient usually showed no marked change until about 3 hours later when it rose to 0.80 or 0.90. During the

TABLE I.

Respiratory Quotients of Rats on a Complete Synthetic Ration.

Weight.	Non-fasting R. Q.	Basal R. Q.	After cane-sugar feeding. R. Q.	After cane-sugar feeding. Time.		After glucose by tube. R. Q.	After glucose by tube. Time.	After glucose by tube. Amount given.
gm.				*hr.*	*min.*		*min.*	*gm.*
284–317	0.82; 0.87	0.78; 0.76	0.82	3		0.90	19	4
			0.90	3				
			0.77	2				
			0.96	8				
310–348	0.84; 0.97	0.73; 0.74	0.97	4	15			
			1.28	4				
			0.95	7				
275–325	0.89; 0.80		0.80	1	25	0.95	50	4
	0.80; 0.91	0.76	1.02	8				

following 3 to 6 hours the quotients were nearly always above 0.90 and frequently considerably above 1.00. Vitamine-deprived animals in a basal condition also consumed cane-sugar set before them and likewise showed a rise in quotient after 3 hours. In two cases quotients of 1.00 and 1.07 were observed in 5 to 6 hours; in one case the figure did not exceed 0.85 at 5 hours, but with this exception there is no indication that the digestion and utilization of cane-sugar suffered any impairment due to lack of vitamine B even when a loss of 28 per cent of the body weight had been produced thereby. A record of the amount of sugar consumed was not always secured because the animals were not in individual cages. In the trials with glucose the animals were separated and in most cases the sugar was given in a concentrated

TABLE II.

Respiratory Quotients of Rats on a Complete Synthetic Ration Followed by Withdrawal of Vitamine B.

Original weight.	Loss in weight.	Non-fasting R. Q.	Basal R. Q.	After cane-sugar feeding.			After glucose by tube.			
				R. Q.	Time.		R. Q.	Time.		Amount given.
gm.	*per cent*				*hr.*	*min.*		*hr.*	*min.*	*gm.*
345		0.81; 0.85	0.73; 0.77	0.79	2	20	0.96		38	4
			0.80	1.00	8					
	10	0.76	0.74							
	11–16	0.79; 0.80	0.75	0.92	5					
	23–24	0.77		0.88	3	20				
210–225		0.88; 0.79; 0.84	0.78; 0.73	1.31	3	30	1.00		40	4
	9–12	0.71	0.77							
	22	0.73	0.70	1.07	5	30				
				0.94	3	25				
200–275		0.82; 0.85	0.73; 0.74	1.13	4					
			0.77; 0.77							
	5–9	0.77; 0.82	0.76							
	14	0.80	0.74	1.00	6					
	19			0.94	3	50				
	24		0.75				0.97	3	20	4.5*
							0.98	19		7 5*
315–355		0.79; 0.80; 0.82	0.72; 0.73	1.21	5		0.81	1	20	3.2
			0.74; 0.77							
	7–15	0.78; 0.81; 0.80	0.72; 0.75	0.83	2	30				
	27	0.76		0.81	3	45				
270–320		0.79; 0.80; 0.82	0.76; 0.77	1.08	5	15				
			0.79							
	13–20	0.87; 0.79	0.78							
	21–27	0.72	0.76	0.82	4					
				0.85	5					
300–365		0.87; 0.88	0.75; 0.74	0.76	1					
			0.73; 0.77	1.01	9					
			0.75; 0.78							
	11–13	0.82	0.73							
	17	0.76	0.75	0.96	3	15				
	28	0.79		0.90	3	5	0.89	2	15	2.5*
							1.02	18		10*
296	41		0.75				0.96	1	30	5.5†
210	40		0.73				0.82		30	4.5
							1.00	3		‡

* Consumed voluntarily; second reading obtained the following morning.

† Died 1 hour later.

‡ Died during the night.

solution by stomach tube. As was to be expected the quotients rose more rapidly than after cane-sugar feeding. Within an hour normal animals gave figures from 0.90 to 1.00. Vitamine-deprived animals required a slightly longer time, but even after losing 40 per cent of their weight, they still showed quotients of 1.00 and 0.96 in $1\frac{1}{2}$ to 3 hours. The administration or glucose in this way was usually followed by diarrhea, diuresis, greatly lowered heat production, and marked prostration in both sets of animals, a prostration which the animals suffering greatest loss in weight by lack of vitamine B did not survive. At the time it was not possible to examine the blood and urine for indications as to the cause of death.

Because of their wide variations none of the figures for total heat production are included in the tables. They were calculated as calories per 24 hours per 100 gm. of weight, also in part as calories per square meter, using the surface area formula $9.1 \times \text{weight}^{\frac{2}{3}}$. The latter figures vary from 1,000 to 2,000 in the extremes, most of them being in the range of 1,400 to 1,700; the animals were obviously not in a resting condition. The corresponding figures for calories per 24 hours per 100 gm. of weight are 18 to 25.

SUMMARY.

From data on the respiratory quotients of rats on rations with and without vitamine B it appears that this substance is not related specifically to the metabolism of carbohydrate since after feeding cane-sugar or glucose the quotients rose in both cases, slightly less rapidly in the vitamine-deprived group due probably to delayed absorption. Basal quotients were alike in both groups, non-fasting quotients were slightly lower in the vitamine-deprived animals, thus reflecting their semifasting condition.

A simple apparatus is described by which, in conjunction with a Haldane apparatus for gas analysis, respiratory quotients can be determined on small animals in 20 to 40 minutes.

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INORGANIC SALT METABOLISM.

II. INORGANIC ION RATIO AFTER ADMINISTRATION OF OXALATES AND CITRATES.

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In a previous paper (1) from this laboratory it has been shown that in parathyroid tetany in dogs there is a marked change in the inorganic ion balance in the blood, especially of potassium and calcium. It became of interest to determine whether the disturbance of one of these ions, produces changes in the other ions similar to that found in parathyroid tetany.

Salant and Wise (2), Salant and Swanson (3), Gates and Meltzer (4), and others have reported that oxalates and citrates produce tetany. They suggest that the tetany is caused by decreased blood calcium, either by increased calcium excretion or by rendering calcium insoluble. The value of soluble calcium salts in oxalate poisoning as a therapeutic measure has long been recognized.

Experiments with both oxalates and citrates have been carried out, but citrates did not produce tetany or change the ionic balance in the concentration used. On the other hand, oxalates produced both tetany and marked changes in the ionic balance.

EXPERIMENTAL.

The procedure of analyses was carried out as described in a previous paper (1), except in the case of magnesium. The magnesium was precipitated in a centrifuge tube, and washed several times with 10 per cent ammonia and finally with ammoniacal alcohol. The procedure from here on was carried out according to the method of Denis (5). When determined by this method somewhat lower results were obtained than when determined

according to the Kramer and Tisdall method (6). These lower results probably represent more nearly the true value for blood. magnesium.

TABLE I.

Effect of Injection of Sodium Oxalate upon the Inorganic Ion Balance of the Blood.

Dog 16. Male. Weight 8.2 kilos.

Per 100 gm. whole blood.						Remarks
Cl_2	P	Ca	K	Na	Mg	
mg.	*mg.*	*mg.*	*mg*	*mg*	*mg*	
310	36.8	5.7	28.4	341.0	2.9	Normal. Apr. 17. 0.615 gm. sodium oxalate by mouth. No symptoms. Apr. 18. 0.615 gm. sodium oxalate by mouth. No symptoms. Apr. 19. 0.82 gm. sodium oxalate by mouth. Vomited. 2.30 p.m. Subcutaneous injection of 0.308 gm. sodium oxalate in 50 cc. water.
243	43.7	4.4	30.6	270.8	3.0	Apr. 20. 9.30 a.m. Subcutaneous injection of 0.615 gm. sodium oxalate. 12.00 m. Distinct tremors. 12.30 p.m. Blood sample. 3.30 " Dog quiet. 10.30 " Subcutaneous injection of 0.41 gm. sodium oxalate.
175	51.3	2.6	42.0	204.2	2.7	Apr. 21. 9.00 a.m. Dog quiet. 10.00 " Subcutaneous injection of 0.615 gm. sodium oxalate. 12.00 m. Tremors; salivation, slight chattering of teeth. 1.30 p.m. Blood sample. Apr. 22. Dog dead.

Dogs which previously fasted for 24 hours were used. We first attempted to administer the material by mouth, but the animals persistently vomited, even when the salt was given in large dilution. Subcutaneous injections of the salts were therefore employed throughout these experiments. Blood samples

were not drawn within 2 hours after injection, which should allow sufficient time for ionic blood balance to become established.

On a few animals electrical reactions were determined as an additional proof of tetany. Considerable difficulty was encountered in making these determinations, so that in other experiments they were not determined.

TABLE II.

Effect of Injection of Sodium Oxalate upon the Inorganic Ion Balance of the Blood.

Dog 17. Female. Weight 16 kilos.

Per 100 gm. whole blood.						Remarks.
Cl_2	P	Ca	K	Na	Mg	
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
300	39.0	5.8	28.6	320	2.7	May 3. Normal.
						" 5.
						2.30 p.m. Subcutaneous injection of 1.6 gm. sodium oxalate in 100 cc. water.
						May 6.
						10.30 a.m. Subcutaneous injection of 0.8 gm. sodium oxalate in 100 cc. water.
						2.00 p.m. Subcutaneous injection of 0.8 gm. sodium oxalate in 100 cc. water.
						6.00 p.m. Dog depressed.
179	43.0	2.5	32.5	188	2.8	May 7.
						10 30 a.m. Subcutaneous injection of 0.8 gm. sodium oxalate in 100 cc. water.
						2.30 p.m. Tremors and twitching, blood sample
						May 8. Large sterile abscess formed at site of injection. Dog killed.

As the changes produced by oxalates are of approximately the same magnitude, Tables I to V will be presented together, with a summary of some of the ratios as calculated.

The balance of the inorganic ions is much more disturbed than that encountered in parathyroid tetany. While in the latter, changes occur chiefly in the calcium and potassium, in the case of oxalates, the only element apparently undisturbed is the magnesium. The most striking result is the marked decrease in both sodium and chlorine. This change is evidently a com-

pensatory shift between acid chlorine cation and the basic sodium anion, as the ratios between the two remain fairly constant.

TABLE III.

Effect of Injection of Sodium Oxalate upon the Inorganic Ion Balance of the Blood.

Dog 18. Female. Weight 13.5 kilos.

Per 100 gm. whole blood.						Remarks.
Cl_2	P	Ca	K	Na	Mg	
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
312	38.6	5.7	29.1	333	3.0	May 16. Normal. Electrical reactions:* A.O.C. 1.9, A.C.C. 1.5, C.C.C. 1.4, C.O.C. —. 12.00 m. Subcutaneous injection of 0.675 gm. sodium oxalate in 100 cc. water. May 17. 1.30 p.m. Subcutaneous injection of 0.675 gm. sodium oxalate in 100 cc. water. 6.00 p.m. Dog all right.
244	43.2	2.8	37.3	250	2.8	May 18. 12.20 p.m. Subcutaneous injection of 0.675 gm. sodium oxalate in 100 cc. water. 3.00 p.m. Tremors and chattering of teeth. 3.30 " Blood sample. Electrical reactions: A.O.C. 0.9, C.O.C. 1.5, A.C.C. 0.8, C.C.C. 0.5.
221	43.0	3.6	36.0	223	2.8	May 19. 10.00 a.m. Dog quite normal. 11.00 " Subcutaneous injection of 0.337 gm. sodium oxalate in 100 cc. water. 6.00 p.m. Slight tremors? Blood sample.
225	47.0	4.6	36.4	238	2.9	May 20. Dog had diarrhea mixed with blood. Blood very dilute; total solids 10.9 per cent. Slight tremors. Blood sample. Dog killed.

* A.O.C. denotes anodal opening contraction; C.O.C., cathodal opening contraction; A.C.C., anodal closing contraction; and C.C.C., cathodal closing contraction.

Total phosphorus and potassium tend to increase in approximately the same proportion. As total phosphorus was determined rather than inorganic phosphates, it is impossible to

state whether the increased phosphorus was wholly inorganic or not. As in our experiments with parathyroid tetany the calcium decreases and the potassium increases. The ranges of the ratio of K:Ca are approximately the same as those in the case of parathyroid tetany. The ratios of the divalent ions to the monovalent ions give results entirely different from those of

TABLE IV.

Effect of Injection of Sodium Oxalate upon the Inorganic Ion Balance of the Blood.

Dog 19. Male. Weight 12 kilos.

Per 100 gm. whole blood.						Remarks.
Cl_2	P	Ca	K	Na	Mg	
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
305	35.5	6.2	29.4	320.0	2.6	June 20. Normal. 3.00 p.m. Subcutaneous injection of 0.6 gm. sodium oxalate in 50 cc. water. June 21. 9.00 a.m. Dog all right. 12.30 p.m. Subcutaneous injection of 0.6 gm. sodium oxalate in 50 cc. water.
270	42.0	3.1	40.2	294.5	2.9	June 22. 10.00 a.m. Subcutaneous injection of 0.6 gm. sodium oxalate in 50 cc. water. 2.30 p.m. Hypersensitive with slight tremors. Blood sample.
250	50.4	3.1	47.6	261.0	2.5	June 23. 11.00 a.m. Subcutaneous injection of 0.6 gm. sodium oxalate in 50 cc. water. 4.00 p.m. Pronounced tremors. Blood sample. June 26. Dog given an injection, but vomited. Discontinued.

parathyroid tetany. There is a strong tendency for this ratio to remain practically unchanged, showing that while there is a marked change in the total ions, there is a tendency for compensatory shifts to occur, so that the resulting ratios remain quite constant. It will be noted that the normal ratios calculated in Table VI are higher than those calculated in a former paper (1), this is due to the lower magnesium values obtained.

Effect of Injection of Sodium Oxalate upon the Inorganic Ion Balance of the Blood.

Dog 20. Female. Weight 8.5 kilos.

Per 100 gm. whole blood.						Remarks.
Cl_2	P	Ca	K	Na	Mg	
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
310	37.0	5.6	28.4	322	3.1	July 5. Normal. Electrical reactions:* A.O.C. 2.0, C.O.C. —, A.C.C. 0.8, C.C.C. 0.8.
205	42.0	4.3	29.5	216	3.2	July 6. 8.00 a.m. Subcutaneous injection of 0.85 gm. sodium oxalate in 100 cc. water. 10.30 a.m. Dog slightly hypersensitive. Blood sample. Electrical reactions: A.O.C. 1.3, C.O.C. 3.8, A.C.C. 1.5, C.C.C. 1.8.
205	51.0	3.8	36 4	207	2.5	July 7. 10.30 a.m. Subcutaneous injection of 0.425 gm. sodium oxalate in 50 cc. water. 3.30 p.m. Distinct tetany (teeth chattering, head movements). Blood sample taken. Electrical reactions: A.O.C. 1.3, C.O.C. 1.6, A.C.C. 1.4, C.C.C. 1.5.
180	45.1	4.9	33.6	208	3.1	July 8. 9.00 a.m. Muscles still twitching, given 15 cc. of 5 per cent calcium lactate solution subcutaneously. 12.00 m. No twitching. Dog brighter. 3.30 p.m. Blood sample. Electrical reactions: A.O.C. 1.6, C.O.C. 4.0, A.C.C. 1.0, C.C.C. 0.8.

* A.O.C. denotes anodal opening contraction; C.O.C., cathodal opening contraction; A.C.C., anodal closing contraction; and C.C.C., cathodal closing contraction.

TABLE VI

Ion Ratios in Tetany after Injection of Sodium Oxalate.

Dog	16	17	18	19	20	Remarks.
$\frac{\text{Sodium}}{\text{Chlorine}}$	1.16	1 07	1.07	1.05	1.04	Normal.
	1.11	1 05	1.07	1.09	1.05	In tetany.
	1.17		1 01	1.04	1.06	" "
			1 05		1.15	" "
$\frac{\text{Potassium}}{\text{Calcium}}$	5.0	4 9	5 1	4.7	5.1	Normal.
	6.9	13.0	13.3	12.9	6.8	In tetany.
	16.1		10.0	15.3	9.5	" "
			7.9		6.8	" "
$\frac{K + Na}{Ca + Mg}$	42.9	41.0	41.6	39.6	40.2	Normal.
	40.7	41.7	51.3	54.0	32.8	In tetany.

Only one attempt was made to relieve the tetany by the injection of calcium lactate. While temporary relief was obtained, analysis shows that calcium was the only element affected. It is difficult to attempt any explanation of such marked changes in the blood salts as are produced by sodium oxalate. Perhaps part of the change may be ascribed to the poisonous action of the oxalate on the various cells of the body. When injections of sublethal doses are continued over a sufficient length of time the animals always die. Gates and Meltzer have demonstrated that oxalates, besides their action on calcium, have a strong toxicological action (4).

Effect of Injection of Sodium Citrate.

Repeated relatively large doses, 1 gm. per kilo of weight, of sodium citrate were ineffectual in either causing a disturbance

TABLE VII

Effect of Injection of Sodium Citrate upon the Inorganic Ion Balance of the Blood.

Dog 21. Female. Weight 15 kilos.

Per 100 gm. whole blood.						Remarks.
Cl_2	P	Ca	K	Na	Mg	
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg*	
305	37.3	Whole blood. 5.6 Serum per 100 cc. 10.3	29.6	338	3.1	Aug. 21. Normal.
310	36.2	Whole blood. 5.8 Serum per 100 cc. 10.2	30.0	345	3.0	Aug. 22. 3.00 p.m. Subcutaneous injection of 70 cc. of 10 per cent sodium citrate solution. Aug. 23. 12.30 p.m. Subcutaneous injection of 70 cc. of 10 per cent sodium citrate solution. No tetany. 4.00 p.m. Blood sample.
308	38.9	Whole blood. 5.7 Serum per 100 cc. 10.9	30.8	332	3.2	Aug. 24. 10.30 a.m. Subcutaneous injection of 70 cc. of 10 per cent sodium citrate solution. 4.00 p.m. No tetany. Blood sample. Discontinued.

of the ionic balance or the production of tetany. Clark (7) has shown that repeated injections of citrates in rabbits temporarily reduce the calcium; however, the analyses with dogs do not show such decreases. Three dogs were studied and all

TABLE VIII.

Effect of Injection of Sodium Citrate upon the Inorganic Ion Balance of the Blood.

Dog 22. Male. Weight 11 kilos.

Per 100 gm. whole blood						Remarks.
Cl_2	P	Ca	K	Na	Mg	
mg.	*mg.*	*mg.*	*mg.*	*mg*	*mg*	
298	36.2	Whole blood. 5.2 Serum per 100 cc. 10.8	28.1	326	2.8	Aug. 28. Normal.
303	35.2	Whole blood. 5.6 Serum per 100 cc. 10.4	27.2	334	3.0	Aug. 29. 10.00 a.m. Subcutaneous injection of 110 cc. of 10 per cent sodium citrate. 2.00 p.m. No tetany. Blood sample.
310	38.5	Whole blood. 5.1 Serum per 100 cc. 10.3	29.9	340	2.6	Aug. 30. Not injected. " 31. 11.00 a.m. Subcutaneous injection of 110 cc. of 10 per cent sodium citrate. 3.00 p.m. No tetany. Blood sample.
305	39.1	Whole blood. 5.3 Serum per 100 cc. 10.6	30.4	329	3.1	Sept. 1. 12.10 p.m. Subcutaneous injection of 55 cc. of 10 per cent sodium citrate. 4.00 p.m. No tetany symptoms. Blood sample. Sept. 2. All right. Discontinued.

presented the same results. While sodium was injected in rather large quantities, at the time of the analyses it had all left the blood stream. As will be seen from Tables VII, VIII, and IX the injections of sodium citrate failed to produce any change in the blood salts.

TABLE IX.

Effect of Injection of Sodium Citrate upon the Inorganic Ion Balance of the Blood.

Dog 23. Male. Weight 14 kilos.

Per 100 gm. whole blood.						Remarks.
Cl_2	P	Ca	K	Na	Mg	
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
300	37.1	Whole blood. 5.6 Serum per 100 cc. 10.5	27.2	331	2.9	Sept. 11. Normal.
295	36.8	Whole blood. 5.8 Serum per 100 cc. 11.0	26.9	340	3.1	Sept. 12. 1.00 p.m. Subcutaneous injection of 140 cc. of 10 per cent solution of sodium citrate. 4.00 p.m. No tetany. Blood sample.
308	38.4	Whole blood. 5.7 Serum per 100 cc. 10.6	28.0	Lost.	3.1	Sept. 13. 10.30 a.m. Subcutaneous injection of 140 cc. of 10 per cent sodium citrate. Sept. 14. 10.30 a.m. Subcutaneous injection of 70 cc. of 10 per cent sodium citrate. 2.30 p.m. No symptoms. Blood sample. Discontinued.

SUMMARY.

1. The subcutaneous injection of sodium oxalate in dogs produces tetany, with a marked disturbance in the blood salts.
2. The sodium, chlorine, and calcium are decreased, while total phosphorus and potassium are increased, magnesium remains singularly constant.
3. The sodium and chlorine decrease in approximately the same proportion, while potassium and phosphorus increase in approximately the same proportion.
4. The subcutaneous injections of sodium citrate failed to produce either tetany or any change in the blood salts.

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A POSSIBLE FACTOR INFLUENCING THE ASSIMILATION OF CALCIUM.*

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Hart and associates (1) have shown that milking goats, when fed a green feed, have been able to utilize the calcium to a higher degree than when fed dry feed. They state that "apparently there is something having its source in fresh green materials, which controls or assists calcium assimilation." Hart and associates (2) have also reported evidence secured with milking cows that it is possible to maintain calcium and phosphorus equilibrium on a ration of grain and dry alfalfa hay—the hay having been cured under caps. These results are contrary to those of Forbes and his coworkers (3) and even to their own later findings (4), when they obtained a negative calcium balance with milking cows on a similar ration. The alfalfa hay in the latter case was cured in windrows for 4 days. They state "these differences in effect of the two alfalfa hays may be attributed to a difference in the degree of destruction during the curing process of the vitamine assisting calcium assimilation."

Working on the hypothesis that most of the calcium, in whatever combination it may be, in the cells of green plants is in a highly dispersed form and hence better assimilated than the calcium in the dry plant, the drying of which no doubt causes a change in the physical properties of the cell and its content, we set about to imitate, in a rough way, the cell content as far as it represents our idea of the highly dispersed form in which the calcium exists in green plants. A starch paste was made up with a known solution of $CaCl_2$ (4 normal). Then an equal volume

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of Na_3PO_4 of the same strength as the $CaCl_2$ was added. The starch acted in a slight degree as a protective colloid for the calcium ion and the final product, $Ca_3(PO_4)_2$, was left in a highly dispersed form. This starch paste was added to the ration of grain and dry timothy hay, which in turn was fed to two milking goats. The grain mixture used consisted of 100 parts ground corn, 100

TABLE I.

Balance of Calcium and Magnesium.

	Record of Goat 5.			Record of Goat 2.		
Date.	Yield of milk per period.	Calcium. Food. Milk. Urine. Feces. Balance.	Magnesium. Food. Milk. Urine. Feces. Balance.	Yield of milk per period.	Calcium. Food. Milk. Urine. Feces. Balance.	Magnesium. Food. Milk. Urine. Feces. Balance.
1921	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Oct. 13 to 20.	6,026	39.46	15.38	5,871	40.27	15.71
		8.29	1.08		6.56	0.99
		4.62	7.69		2.50	6.13
		26.36	11.35		26.66	12.19
		+0.19	−4.74		+4.55	−3.60
Oct. 20 to 27.	5,823	38.66	15.05	5,446	40.27	15.71
		7.16	1.06		5.82	0.94
		3.39	7.18		2.27	6.18
		24.01	10.77		31.02	13.90
		+4.10	−3.96		+1.16	−5.31
Oct. 27 to Nov. 8.	10,585	62.31	24.60	9,093	55.31	22.55
		12.14	2.04		9.39	1.70
		6.41	11.82		2.46	9.48
		44.08	17.82		41.03	18.08
		−0.32	−7.08		+2.43	−6.71

parts ground oats, 50 parts wheat bran, and 30 parts linseed oil meal. The proportion of hay to the grain mixture as fed was about as 1 is to 1½. No salt was given as such since it was formed in the reaction between the calcium chloride and the sodium phosphate.

The goats were mature animals in the beginning of their 4th (No. 5) and 5th (No. 2) months of lactation, respectively, and gave a milk yield of from 700 to 850 gm. per day. None of the

goats was bred. They were confined in the metabolism crates designed by Dr. Forbes for hogs. The test was carried on for a period of 26 days, preceded by a preliminary period of 10 days. The 26 day period was divided into three periods of 7, 7, and 12 days, respectively. The goats were milked and fed twice daily. The excreta were collected quantitatively. The methods used for the determination of calcium and magnesium were the same as those given in Ohio Agricultural Experiment Station Bulletin 363. All determinations were made in triplicate. The results are recorded in Table I.

DISCUSSION.

Our results show (Table I) that five of the six complete accountings of the calcium were positive or in equilibrium, while one was negative. This negative balance may be due to the fact that the animal, No. 5, refused to eat the last 288 gm. of mixed feed. Part of this had been accumulating for a day or two and, no doubt, had lost some moisture. When this was analyzed and subtracted from the apparent positive balance, a negative balance was the result. The magnesium balances were all negative. This may be due to the physiological antagonism between calcium and magnesium or it may be due to an insufficient intake. The results as far as the calcium balances go were a little surprising to us, especially after we had failed in a previous trial which will be reported later, to obtain a positive calcium balance on a dry ration and a mineral supplement. We realize that the animals were in a later stage of lactation in this trial than in the one 2 months previous when a negative calcium balance was obtained, with practically the same intake, and this may be the factor affecting our results, but the data presented here raise a question that we believe is worth some consideration.

The cow may be a more highly specialized animal than the milking goat, and whether similar or identical results can be obtained with milking cows remains to be seen.

A vitamine, or the vitamines, resident in green plants and properly cured alfalfa hay, as reported by Hart, may play an important part in the assimilation of calcium, yet we believe the difference can be partly attributed to the difference in the physical properties of the cell wall and cell content of green and dry hay and, there-

fore, a difference in the digestibility. It is true that drying causes a reversible or irreversible change in an artificial colloidal system, depending upon the presence of a protective colloid, and we have reasons to believe that a similar change takes place in the plant when it dries, and if such is the case then the highly dispersed materials coalesce and form larger aggregates and these large aggregates may be less permeable to the digestive juices than the highly dispersed material and, therefore, not so readily digested and assimilated. It is not our intention to leave the impression that this is the only difference in the green and dry plant or that this is the only factor in causing the difference in the assimilation of calcium. If our results are only the effect of adding a calcium supplement then they are contrary to those of Forbes and coworkers (3), Hart and associates (4), and our unpublished data.

SUMMARY AND CONCLUSIONS.

1. The calcium balance in two milking goats was favorably influenced by feeding tricalcium phosphate, which was precipitated in a starch paste. This influence may be due to the fineness of division of the tricalcium phosphate.

2. The results of this experiment seem to indicate that possibly the difference between green and dry hay in causing a difference in the assimilation of calcium is partly due to the difference in the physical properties (fineness of division) of the cell content of the two hays and, therefore, a difference in the digestibility.

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LYSOLECITHINS AND LYSOCEPHALINS.

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Through the action of cobra venom on lecithin, Kyes,[1] working in Ehrlich's laboratory, obtained a substance possessing strong hemolytic properties. Ehrlich and Kyes regarded the substance as a combination of lecithin and cobra venom. Manwaring[2] later questioned the correctness of the conclusions of Ehrlich and Kyes regarding the chemical nature of the hemolytic substance discovered by them, and suggested that it was a product of partial hydrolysis of lecithin. Wilstätter and Lüdecke[3] later advanced definite evidence in favor of the latter theory. Wilstätter and Lüdecke further demonstrated that the product formed by the action of cobra venom differed from lecithin by the absence in its molecule of the radical of unsaturated acid. Finally, Delezenne and Fourneau[4] succeeded in preparing from the products of the reaction of cobra venom on lecithin a crystalline substance which had the elementary composition of palmityl lecithin and which they named lysocithin.

The substance described by Delezenne and Fourneau was prepared by the action of cobra venom directly on egg yolk, and not on isolated lecithin. Wilstätter and Lüdecke also worked with crude lecithin, which undoubtedly contained a considerable proportion of cephalin. Thus it seemed as if cobra venom had a selective action on lecithin, and if this had been so, it might have led to a method for freeing cephalin from its mixture with lecithin,

[1] Kyes, P., *Biochem. Z.*, 1907, iv, 99; 1908, viii, 42; *J. Infect. Dis.*, 1910, vii, 181.

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and in this way to a method of preparation of pure cephalin. Thus the question of the degree of specificity of the cobra venom enzymes acquired practical importance.

From another view-point, also, the product of the action of cobra venom on lecithin required reinvestigation. At the time of the work of Delezenne and Fourneau, it was generally believed that there existed only one form of lecithin. The more recent work of this laboratory[5,6] has made it evident that there exist several forms of lecithin; some containing in their molecule a palmitic, others a stearic, acid radical. In the light of this knowledge, it was natural to expect two forms of lysolecithin. Because of all these considerations we concluded to reinvestigate the composition of the product obtained by the action of cobra venom on egg yolk.

As was to be expected, the product of partial hydrolysis of a mixture of lecithin and cephalin is as complex as the original mixture. In the partially hydrolyzed material, all the unsaturated fatty acid radicals were missing, the other components of a mixture of lecithin and cephalin were still present. On complete hydrolysis of the material two fatty acids, palmitic and stearic, and two bases, choline and amino ethanol, were identified. Thus the product was a mixture of several substances, which we propose to name lysolecithin and lysocephalin. Inasmuch as lecithins differ in the nature of their saturated fatty acids, it is probable that there exist several lysolecithins. As yet no attempt was made at a systematic fractionation of the individual substances, but fractions were already obtained which differ considerably in the relative proportion of lysocephalin and lysolecithin. The lowest proportion of the former was 7 per cent and the highest 33 per cent.

Thus far it has not been possible to separate lysolecithin from lysocephalin by means of cadmium chloride. The nitrogen distribution in the cadmium chloride compound remained the same as in the original material.

Preliminary tests on the hemolytic action of various fractions showed no differences in their activity.

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EXPERIMENTAL.

48 dozen eggs were suspended in their own volume of $\frac{M}{15}$ phosphate solution (pH = 7.5). To every 8 liters of such a solution 0.250 gm. of cobra venom suspended in a small quantity of phosphate solution and 1 cc. of toluene were added and the material digested at 40°C. for 16 hours. At the end of this period the addition of an equal volume of acetone precipitated 80 per cent of the hydrolyzed phosphatides present together with all the proteins. This precipitate (A) was exhaustively extracted with hot 95 per cent alcohol and the solution, after concentration to a very small volume, was poured into 10 liters of acetone. The crude precipitate (B) was dissolved in a minimal quantity of warm alcohol and precipitated with 10 liters of ether. This material, 325 gm., analyzed as follows:

No. 66. 0.1036 gm. substance: 0.0946 gm. H_2O, 0.2170 gm. CO_2, and 0.0154 gm. ash.
0.1880 gm. substance: (Kjeldahl) 3.75 cc. 0.1 N acid.
0.2821 " " : (fusion) 0.0584 gm. $Mg_2P_2O_7$.

In all the samples the nitrogen distribution was determined by hydrolyzing 1 gm. of substance with 150 cc. of 10 per cent hydrochloric acid. After neutralization the solution was concentrated to 10 cc. and filtered. Of the resulting solution 5 and 2 cc. were used to determine, respectively, its total nitrogen and amino nitrogen content.

5 cc.: (Kjeldahl) 5.00 cc. of 0.1 N acid.
2 " : (Van Slyke) 1.33 " N_2 at 20°C., 752 mm.
Found. No. 66. C 57.11, H 10.21, N 2.79, P 5.77, ash 14.86.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{26}{100}$$

Calculated. Lysolecithin (containing palmitic acid).
$C_{24}H_{52}O_8PN$. C 56.10, H 10.29, N 2.73, P 6.04.

Lysolecithin (containing stearic acid).
$C_{26}H_{56}O_8PN$. C 57.65, H 10.43, N 2.59, P 5.73.

Lysocephalin (containing stearic acid).
$C_{23}H_{48}O_7PN$. C 57.35, H 10.05, N 2.91, P 6.44.

It absorbed no iodine (Wijs method).

Observation of its optical rotation, taken in chloroform solution, gave:

$$[\alpha]_D^{20} = \frac{-0.14° \times 100}{1 \times 10} = -1.4°$$

This material melted at 212°.

On cooling to −21°C., the mother liquor from (B) deposited a voluminous precipitate. After solution in alcohol and precipitation with ether, this material [No. 67 (30 gm.)] analyzed as follows:

0.1028 gm. substance: 0.2222 gm. CO_2, 0.0952 gm. H_2O, and 0.0146 gm. ash.
0.1869 gm. substance: (Kjeldahl) 3.45 cc. 0.1 N acid.
0.2804 " " : (fusion) 0.0562 gm. $Mg_2P_2O_7$.
After hydrolysis: 5 cc.: (Kjeldahl) 5.20 cc. 0.1 N acid.
2 " : (Van Slyke) 0.38 cc. N_2 at 20°C., 752.7 mm.
Found. No. 67. C 58.94, H 10.36, N 2.58, P 5.58, ash 14.22.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{7}{100}$$

No. 67 absorbed no iodine.

In chloroform solution its rotation was

$$[\alpha]_D^{20} = \frac{-0.13° \times 100}{1 \times 10} = -1.3°$$

It melted at 175°.

From the mother liquor (A) an additional 60 gm. of material was recovered by precipitation as the cadmium chloride salt and subsequent recovery of the free bases. It was not purified further, but analyzed as follows:

No. 75. 0.1010 gm. substance: 0.2310 gm. CO_2, 0.0982 gm. H_2O, and 0.0120 gm. ash.
0.1895 " " : (Kjeldahl) 3.55 cc. 0.1 N acid.
0.2843 " " : (fusion) 0.0484 gm. $Mg_2P_2O_7$.
After hydrolysis: 5 cc.: (Kjeldahl) 2.90 cc. 0.1 N acid.
2 " : (Van Slyke) 0.49 " N_2 at 22°C., 764.4 mm.
C 62.37, H 10.87, N 2.62, P 4.74, ash 11.88.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{16}{100}$$

The mixture of lysolecithin and lysocephalin may be precipitated from alcoholic solution by cadmium chloride. The

mixed cadmium salts form a white, non-crystalline powder, soluble in water, dilute alcohol, and dilute ether, from which solvents it again separates as an amorphous powder. It is insoluble, however, in most organic solvents. As is the case with the cadmium chloride salt of lecithin itself, these derivatives also do not contain the inorganic salt in molecular proportions.

No. 71. 0.1979 gm. substance: (Kjeldahl) 2.55 cc. 0.1 N acid.
0.2968 " " : (fusion) 0.0416 gm. $Mg_2P_2O_7$.
0.2968 " " : 0.1030 gm. CdS.
0.2032 " " : (Carius) 0.1056 gm. AgCl.
After hydrolysis: 5 cc.: (Kjeldahl) 2.10 cc. 0.1 N NaOH.
2 " : (Van Slyke) 0.52 " N_2 at 26°C., 758.7 mm.
Found. No. 71. N 1.80, P 3.90, Cd 18.71, Cl 12.85.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{24}{100}$$

The mixed cadmium chloride salts may be freed from their inorganic constituent by treating the suspension of the salts in toluene with methyl alcoholic ammonia. After the solvents were removed under diminished pressure, the residue was dissolved in a small volume of absolute alcohol and filtered. The material which was precipitated by the addition of ether analyzed as follows:

0.1008 gm. substance: 0.2140 gm. CO_2, 0.0944 gm. H_2O, and 0.0150 gm. ash.
0.0940 " " : (Kjeldahl) 1.85 cc. 0.1 N acid.
0.1880 " " : (fusion) 0.0396 gm. $Mg_2P_2O_7$.
After hydrolysis: 5 cc.: (Kjeldahl) 2.40 cc. 0.1 N acid.
2 " : (Van Slyke) 0.81 " N_2 at 26°C., 758.7 mm.
Found. No. 70. C 57.89, H 10.48, N 2.75, P 5.88, ash 14.88.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{33}{100}$$

The Fatty Acids Present in the Lysolecithin and Lysocephalin Mixture.

100 gm. of the mixture of lysolecithin and lysocephalin (No. 66) were hydrolyzed by boiling for 8 hours with 10 per cent hydrochloric acid. The mixture was cooled and filtered (C) and the fatty acids were dissolved in ether and thoroughly washed with

water. After concentration, the residue (58 gm.) was esterified and the mixed methyl esters were fractionated by distillation at a pressure of 1.5 mm.

The esters were saponified, and analyses, melting points, and molecular weight determinations were made on the free acids in the manner described in previous publications.[5]

No. 81. 0.1005 gm. substance: 0.2764 gm. CO_2 and 0.1154 gm. H_2O.
1.0744 " " required for neutralization 8.40 cc. 0.5 N NaOH.

No. 82. 0.1000 gm. substance: 0.2740 gm. CO_2 and 0.1114 gm. H_2O.
1.0402 " " required for neutralization 8.0 cc. 0.5 N NaOH.

No. 83. 0.0997 gm. substance: 0.2766 gm. CO_2 and 0.1124 gm. H_2O.
1.0064 " " required for neutralization 7.55 cc. 0.5 N NaOH.

No. 84. 1.0010 gm. substance required for neutralization 7.6 cc. 0.5 N NaOH.

No. 85. 0.7597 gm. substance required for neutralization 5.7 cc. 0.5 N NaOH.

No. 86. 0.0998 gm. substance: 0.2762 gm. CO_2 and 0.1134 gm. H_2O.
1.1270 " " required for neutralization 8.10 cc. 0.5 N NaOH.

No. 87. 0.1002 gm. substance: 0.2790 gm. CO_2 and 0.1132 gm. H_2O.
0.8922 " " required for neutralization 6.3 cc. 0.5 N NaOH.

Nos. 81, 82, and 83 were the redistilled fractions of the lowest boiling fraction.

	No	Boiling point of ester at 1.5 mm.	Weight of ester	Analysis of acid		Molecular weight of acid	Melting point of acid.
				C	H		
		°C.	gm.				°C.
Found.	81	167-170	3	74.99	12.84	255	62-63
"	82	172	3	74.72	12.49	260	57-58
"	83	172-175	6	75.65	12.61	266	58-58.5
"	84	171-176	10			270	57-58
"	85	168-177	12			275	66-68
"	86	175-181	6	75.47	12.71	278	67-68
"	87	180-190	3	75.93	12.64	283	71-71.5
Calculated.	$C_{16}H_{32}O_2$			74.92	12.58	256	63
"	$C_{18}H_{36}O_2$			75.98	12.76	284	71

The bases were isolated from the filtrate (C) of the original hydrolysis liquor by the method described by Levene and Ingvaldsen.[6] A gold salt was obtained from the amino ethanol fraction which melted at 189–190°C. (uncorrected) and gave the following analysis:

0.1015 gm. substance: (ignition) 0.0497 gm. Au.

$C_2H_6ONAuCl_4$.	Calculated.	Au 49.17.
	Found.	" 48.96.

From the choline fraction a picrate was precipitated which melted at 241–242°C. (uncorrected). This was decomposed with dilute hydrochloric acid and extracted with ether. The aqueous liquor was concentrated to dryness and the residue in alcoholic solution precipitated with platinic chloride. This salt decomposed at 243°C. (uncorrected).

0.1012 gm. substance: (ignition) 0.0321 gm. Pt.

$(C_5H_{14}ONCl)_2PtCl_4$.	Calculated.	Pt 31.64.
	Found.	" 31.81.

A METHOD FOR THE DETERMINATION OF DISSOLVED CARBON DIOXIDE.

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(Received for publication, February 13, 1923.)

The determination of carbon dioxide, liberated in respiration experiments by aquatic animals, is especially difficult and uncertain. This is due partly to the fact that the quantity liberated is only a small fraction of the total quantity dissolved in the water in the form of carbonates, bicarbonates, and free carbon dioxide, and partly because the animals experimented on are often likely to give off substances which may interfere with the results obtained in many of the methods for the determination of carbon dioxide. It is known that urine, feces, and mucus will yield some carbon dioxide when the water in which they have been excreted is boiled after acidification. Some observers have also noted that acids may be given off by some aquatic animals, fish for example, in such amounts as to interfere with the results obtained by titration methods (Krogh, 1916).

Morgulis and Fuller (1916) have shown that the titration methods for the determination of carbon dioxide, especially by using phenolphthalein as an indicator, give a wide range of error. They conclude that the results obtained by such methods are of questionable value and no accurate conclusions can be derived from them. Johnston (1916) states similar objections.

The vacuum extraction principle has certain advantages over that of many of the other methods employed for the determinations of dissolved gases. (1) The relatively short time within which an equilibrium is attained between the gas content of water and the atmosphere; (2) the gases are obtained and may be directly measured volumetrically; (3) there is a non-interference of metabolic products.

Apparatus.

The apparatus used in the determination of free and combined carbon dioxide in water consists in general of three parts: an evacuation tube, a measuring burette, and an absorption pipette (Fig. 1). The evacuation tube is constructed on the principle of the well known Van Slyke blood gas apparatus (Van Slyke, 1917; Van Slyke and Stadie, 1921). Because of the large amount of the sample necessary to yield sufficient carbon dioxide for accurate determination the Van Slyke apparatus cannot be used for dissolved carbon dioxide in water satisfactorily. As in the Van Slyke apparatus a Torricellian vacuum is produced by a column of mercury. The apparatus is constructed of strong glass in order to withstand the weight of the mercury without danger of breaking. A cup, *A*, to which is attached a three-way stop-cock, *1*, is calibrated into 10 cc. divisions, the total capacity being approximately 100 cc. Below this cup is the evacuation pipette, *B*, with a capacity of 250 cc. The mercury is drawn from the pipette through a three-way stop-cock, *2*, by way of *C′* or *C*. *D* serves as a receptacle for the mercury having a capacity of about 500 cc. A fine bore burette, *F*, is calibrated into 0.01 cc. divisions. The total capacity is about 5 cc. This burette is surrounded by a large glass tube, *G*, for the purpose of maintaining a constant temperature during the analysis. A mercury receptable, *L*, is used for levelling the height of the mercury in the burette. Between the evacuation pipette and the burette is inserted a three-way stop-cock, *3*. The outlet, *K*, serves as a means of emptying the evacuation pipette and also in enabling the operator to fill the tubes *N* and *N′* with greater ease. An absorption pipette, *P*, filled with 10 per cent potassium hydroxide is used for the absorption of the carbon dioxide. A fine bore tube, *H*, enables the operator to level accurately the mercury in the measuring burette.

Method.

The procedure for the determination of carbon dioxide is as follows: The air in the tube, *H*, is made carbon dioxide-free by absorption in the alkali, by methods that are commonly used in gas analysis apparatus. The potassium hydroxide is brought to

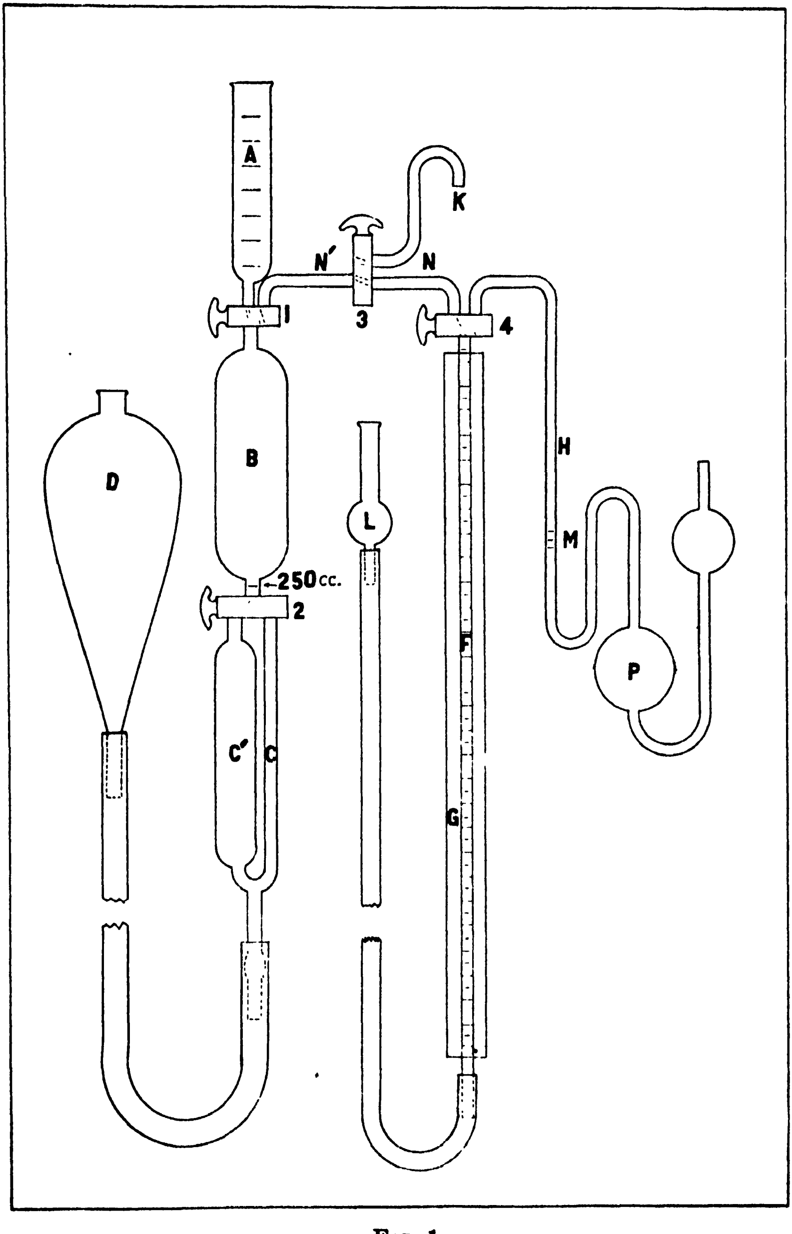

FIG. 1.

the mark, *M*. Stop-cock *4* is then turned so as to allow the mercury to rise to stop-cock *3*. The evacuation pipette, *B*, is then filled with mercury, stop-cock *3* is turned so as to allow a small amount of mercury to rise in outlet tube *K*. The apparatus is then ready for the introduction of the sample.

25 or 50 cc. of the water to be analyzed are introduced into the cup, *A*, by using the calibration or by pipette. A pipette is usually found the more practicable. Where the free carbon dioxide is high in amount a few drops of paraffin oil are first placed in the cup which forms a thin film on the surface of water, preventing to a large extent the escape of gas. The sample is slowly drawn into the evacuation pipette by lowering the mercury receptacle *D*, the oil remaining in the cup. Stop-cock *1* is tightly closed and the evacuation begun. The mercury is lowered in the evacuation pipette to the 250 cc. mark. After standing for about a minute the water is trapped in *C′* and the mercury allowed to rise in the pipette through *C*. The gas liberated is allowed to displace the mercury in the tubes *N* and *N′*. When the total carbon dioxide determinations are to be made 10 cc. of 10 N sulfuric acid are introduced, following the sample, and evacuation proceeds in a similar manner to that described.

Several evacuations are usually necessary to liberate completely all of the carbon dioxide present; ten will suffice in most cases. Shaking is not practical with such a large apparatus. Repeated evacuations appear, however, to be as efficacious, especially when the sample is trapped in *C′* and the pipette *B* filled from *C*. After the gas liberated has been expelled the mercury is drawn back through *C′* in a fine spray. This greatly increases the surface of the liquid exposed, thus hastening the liberation of the gases.

When all the gas has been expelled and collected in the burette the mercury is raised so as to flow into the tube *N*, forcing the gas into the burette *F*, none remaining in the tube. The stopcock *4* is then turned, connecting the burette with the absorption pipette *P*, care being taken that the mercury in the levelling tube *L* is very nearly the same level as that in the burette. The potassium hydroxide is brought exactly to the point *M*. A reading is then made on the burette. The gas is then forced over into the absorption pipette several times. When a constant

reading is obtained the amount absorbed is determined. This represents the volume of carbon dioxide liberated from the sample at a given temperature, pressure, and vapor tension. The air is kept saturated with water vapor. The volume is reduced to standard conditions by the formula:

$$V_0 = \frac{(B - p)\ V}{760\ (1 + 0.003667\ t)}$$

V_0 is the volume of dry gas at 0°C. and 760 mm., B being the barometric pressure, p the pressure of aqueous vapor at t°C., V the volume of the gas at t° and the observed pressure, and 0.003667 the coefficient of cubic expansion of gases.

The apparatus described serves as a convenient and an accurate means of determining the amount of carbon dioxide dissolved in water. It is especially useful in the analysis of water during respiration experiments and in other analyses where titration methods appear inadequate.

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ON THE MECHANISM OF PHLORHIZIN DIABETES.

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The characteristic hypoglycemia in phlorhizin diabetes has, from the earliest investigations of this condition, emphasized the kidney involvement and, unquestionably, has prejudiced the interpretation of numerous experimental results which might be taken to implicate other factors. While, from time to time, a less restricted mechanism has been suggested, as by Underhill (1), the view still prevails rather generally that the failure of the phlorhizinized animal to burn glucose is due to the subnormal concentration of glucose in the tissues, resulting from a lowered renal threshold for sugar. Perhaps Allen (2) has stated the substance of this view most positively:

". . . . phloridzin produces no intrinsic impairment of utilization. Phloridzin compels the excretion of more or less dextrose through the kidneys; but when this demand is fully satisfied, the phloridzinized animal is able to utilize sugar just as readily as any other animal . . . . the paradoxical law of dextrose holds good in phloridzin poisoning just as in all other non-diabetic conditions . . . . the more sugar is given, the more is assimilated, just as in the normal animal. This rule applies even in the so-called 'maximal' phloridzin poisoning. . . . . The 'maximally' phloridzinized dog will excrete quantitatively all sugar up to a certain amount; but any quantity above this dose will be assimilated just as in the normal animal. The impression given is as though some compound in the body of the phloridzinized animal requires saturation with dextrose; this compound is broken up by the kidney and the dextrose excreted; but the power of utilizing dextrose as such is absolutely unchanged, and accordingly any dextrose in excess of the amount required for the hypothetical compound is utilized in normal manner. . . . . Dextrose does not circulate in free form in the phloridzinized organism. In phloridzin poisoning, we see the paradox that an animal, which constantly excretes dextrose spontaneously, is at the same time able to utilize injected dextrose; and the quantity utilized increases with the dose injected, just as in the normal animal."

Erlandsen (3) believes that there is no ground for supposing an extrarenal phlorhizin effect, other than the indirect effect of the diminishing concentration of dextrose in the blood.

Stiles and Lusk (4) accept Loewi's (5) theory that the blood sugar is normally in a loose combination with colloid substance, and add to this theory the hypothesis that the colloid sugar compound cannot be burned. They conceive that in phlorhizin glycuresis the kidneys break up the colloid sugar, and the sugar may then be eliminated. These authors state further, "There is, therefore, a 'total phlorhizin diabetes' within certain limits, but these limits must not be overstepped by flooding the organism with sugar. In this latter case sugar can be burned."

Later, when Ringer (6) was able to recover in the urine of a phlorhizinized dog as large a dose as 75 gm. of glucose, Lusk (7) apparently reached the conclusion that the completely phlorhizinized dog has lost the power of oxidizing sugar. Lusk himself found that the ingestion of this quantity of glucose by a phlorhizinized dog had no effect upon the respiratory quotient; and suggested that the failure to burn any sugar may be due to the development of acidosis.

Ringer (6), commenting on the experiments of Minkowski (8), who found no increase in the blood sugar concentration after nephrectomy in phlorhizinized dogs, says: "If the kidneys were not the only seat of attack of the phlorhizin, and if phlorhizin *per se* had any influence on the sugar burning capacity of the cells of the body, nephrectomy should have been followed by a rise in the glucose concentration of the blood. . . . . "

Both Underhill (1) and Csonka (9) found marked increases in the blood sugar concentration of phlorhizinized dogs after ligation of the renal structures. Underhill concluded that phlorhizin also exerts an action upon other structures than the kidney, resulting in the increased production of sugar.

Csonka (9) also observed in a phlorhizinized dog a hyperglycemia of about 3 hours duration following the feeding of 20 gm. of glucose; and in a second dog, after feeding 50 gm. of glucose, a hyperglycemia which was maintained for about 8 hours. Csonka does not show, however, the quantitative elimination of the glucose fed in these two cases.

The finding of a hyperglycemia in phlorhizinized animals, following nephrectomy, can have no conclusive bearing upon the study of the mechanism of phlorhizin glycuresis unless it is shown that an increased blood sugar concentration does not result from nephrectomy in an otherwise normal subject. It is quite possible that the increased reducing substance found in such cases may not be sugar; terminal nephritis in humans practically always shows a high blood sugar as estimated by available methods, but there is not satisfactory evidence that the high figure obtained is due to sugar. Underhill's experiments are open to the criticism

of failure to employ controls of non-phlorhizinized, nephrectomized animals. We have found but one recorded experiment thus controlled; Csonka (9) observed no appreciable change in the blood sugar content of a normal dog following the ligation of the renal vessels and ureters, even when 50 gm. of dextrose were ingested. If this single result is accepted as typical, the hyperglycemia noted in phlorhizinized, nephrectomized animals may be interpreted as indicating an extrarenal effect of phlorhizin. Obviously, however, these experiments do not show whether the hyperglycemia is attended by sugar utilization, and, hence, neither confirm nor disprove the theory that the phlorhizinized animal is able to burn carbohydrate when it is present in sufficient concentration.

We believe that a decisive test of this theory is to ascertain whether dextrose ingested by a phlorhizinized dog in amount sufficient to maintain a prolonged hyperglycemia is quantitatively recovered in the urine. With this view we have carried out the experiments reported herein.[1]

In two cases we have fed a mixture of urea and dextrose. The urea was included for purposes of a separate study[2] of surprising variations in the ratios of dextrose to nitrogen which repeatedly have been observed in this laboratory in the urine of phlorhizinized dogs. The results of including the urea, disclose also a relationship in the excretion of sugar and urea to which we have called attention in the present paper.

GENERAL PROCEDURE.

Female dogs were employed in the experiments. These animals were fasted throughout the experimental period, and, beginning on the 3rd day of starvation, received subcutaneous injections of phlorhizin in olive oil at intervals as shown in the tables.

Each urine was separately and automatically collected from the cage and the time at which it was voided was recorded, by an apparatus[3] constructed in this laboratory.

[1] A preliminary report of this work was made by Guion and Benedict (10).

[2] This study is still in progress, but it is hoped to report upon it at an early date.

[3] A description of this apparatus, which was designed by Mr. C. Dudley, is contemplated in a subsequent publication.

Duplicate analyses were made of each urine sample for total nitrogen, by the macro Kjeldahl procedure, and for total reducing sugar, by the Allihn method.

Blood for analysis was taken from the jugular vein through a needle inserted through the skin. Potassium oxalate was added to prevent clotting. Urea nitrogen was estimated by a urease-aeration-nesslerization procedure, employing 2 cc. of blood; and blood sugar was determined by the Benedict modification of the Lewis-Benedict method.

Merck's phlorhizin, Kahlbaum's urea, and a C.P. grade of anhydrous dextrose were used.

EXPERIMENTAL.

Dog 36.—In the 24 hour urine of this dog for the 5th day of starvation and the 3rd day of phlorhizin, the D:N ratio was quite constant with an average value of 3.50. The urine of the following 2 hour period, from 7.00 to 9.00 a.m., Apr. 4 (Table 1), showed a ratio of 3.54. 15 minutes later the dog received by stomach tube a mixture of 30 gm. of dextrose and 17.7 gm. of urea in 300 cc. of warm water. In this mixture the ratio of dextrose to nitrogen was 3.63. The complete data of the subsequent urine and blood findings are given in Table I. It will be noted that a very marked hyperglycemia follows the ingestion of the dextrose mixture, and endures for about 6 hours. At the same time, the blood urea increases to an even greater degree above the control level; and we take this fact as evidence that the ingested urea is absorbed at least as rapidly as the dextrose. At the end of 6 hours the blood sugar has returned to the control level, while the blood urea is still double its initial value.

The blood sugar and urea nitrogen curves parallel very closely the hourly excretion of sugar and nitrogen in the urine. During the first 3 hours after ingesting the dextrose-urea mixture the rate of excretion of sugar mounts rapidly; during the 3rd hour the rate is nearly five times that of the control period. It is not until the 6th hour that sugar elimination approaches again a rate attributable to the basic endogenous metabolism. In the same time the rate of nitrogen excretion has also greatly increased, but not so rapidly; and the subsequent fall in this rate is less sharp than in the case of sugar, approaching the level of the control period not until the 11th hour. The resulting urinary D:N values are, therefore, higher than normal during the first 3 hours, and lower than normal during the succeeding 3 hours, after which there is a slow return to the original ratio.

TABLE I.

Dog 36. Female. Weight, 12.2 kilos. Last feeding, Mar. 29. Beginning Apr. 1, 1.0 gm. of phlorhizin in olive oil was injected subcutaneously at 6.00 p.m. daily.

Date.	Urine. Time of collection.	Urine. Volume.	Urine. Total N		Urine. Total sugar.		Urine. D:N	Jugular vein blood. Urea N per 100 cc.	Jugular vein blood. Sugar per 100 cc.	Remarks.
		cc.	N per hr.	gm.	sugar per hr.	gm		mg	mg.	
Apr. 3	6.35 a.m. to 4.45 p.m.	240	0.52	5.27	1.85	18.80	3.57			
	9.10 "	197	0.50	2.23	1.77	7.83	3.51			
" 4	7.00 a.m.	245	0.51	4.98	1.74	17.06	3.42			
Total	....	682		12.48		43.69	3.50			

At 9.15 a.m., Apr. 4, given by stomach tube a mixture of 30 gm. of dextrose and 17.7 gm. of urea (8.26 gm. of nitrogen) in 300 cc. of warm water. In this mixture D:N = 3.63.

Date.	Urine. Time of collection.	Urine. Volume.	Urine. Total N		Urine. Total sugar.		Urine. D:N	Jugular vein blood. Urea N per 100 cc.	Jugular vein blood. Sugar per 100 cc.	Remarks.
		cc.	N per hr.	gm.	sugar per hr.	gm		mg	mg.	
Apr. 4	9.00 a.m.	20	0.46	0.91	1.61	3.22	3.54	14	86	
	11.30 "	295	1.47	3.68	6.38	15.96	4.33	66	242	
	12.55 p.m.	295	1.79	2.54	7.67	10.86	4.27			
	1.15 "							46	166	
	3.05 "	190	0.83	1.79	2.25	4.87	2.72	27	86	
	4.25 "	180	1.16	1.54	2.60	3.47	2.25			
	11.25 "	230	0.51	3.55	1.49	10.42	2.93			
" 5	6.15 a.m.	160	0.45	3.10	1.58	10.82	3.49			
	9.30 "	50	0.45	1.45	1.56	5.07	3.49			
Total	....	1,420		18.56		64.69	3.37			
Apr. 5	9.30 a.m. to									
" 6	9.30 a.m.	801		11.23		38.53	3.43			

Of the 30 gm. of dextrose ingested by this dog, 95.5 per cent was recovered in the subsequent 24 hour urine as "extra" sugar. The calculation was made in the usual manner. This involves

TABLE II.

Dog 34. Female. Weight, 8.7 kilos. Last feeding, Apr. 20. Beginning Apr. 22, 1.0 gm. of phlorhizin in olive oil was injected subcutaneously at 6.00 p.m. daily.

Date.	Urine.							Jugular vein blood.	Remarks.
	Time of collection.	Volume	Total N.		Total sugar.		D:N.	Sugar per 100 cc.	
		cc	N per hr	gm	sugar per hr	gm		mg	
Apr. 24	8.35 a.m. to 12.35 p.m.	40	0.27	1.08	1.01	4.04	3.74		
	4.20 "	39	0.30	1.13	0.93	3.48	3.08		
	7.25 "	42	0.31	0.97	1.30	4.02	4.14		
	11.05 "	50	0 29	1.05	1.02	3.75	3.57		
" 25	2.45 a.m.	60	0.29	1.08	1.09	3.99	3.69		
	7.45 "	58	0.29	1.46	1.04	5.20	3.56		
	9.20 "	18	0.30	0.48	1.08	1.71	3.56		Urine taken by catheter.
Total		307		7.25		26 19	3.61		

At 9.20 a.m., Apr. 25, given by stomach tube 30 gm. of dextrose in 250 cc. of warm water.

Date.	Time of collection.	Volume	N per hr	gm	sugar per hr	gm	D:N.	Sugar per 100 cc.	Remarks.
Apr. 25	9.10 a.m.							68	
	11.05 "	87	0.33	0.58	4.45	7.79	13.43		
	11.20 "							201	
	1.15 p.m.	142	0.25	0.55	5.31	11.50	20.91	202	
	3.20 "	128	0.23	0.48	4.99	10 41	21.69	143	
	5 30 "	66	0.20	0.43	1.72	3.72	8.65		Last injection of phlorhizin, 6.00 p.m.
	8.30 "	62	0.22	0.65	0.92	2.75	4.23		
	11.05 "	60	0.23	0.61	0.87	2.26	3.70		
" 26	1.35 a.m.	36	0.22	0.57	0.86	2.14	3.75		
	6.10 "	82	0.24	1.11	1.00	4.58	4.12		
	8.20 "	32	0.25	0.53	0.78	1.68	3.17		
	10.45 "	39	0.30	0.72	1.01	2.45	3.40		
Total		734		6.23		49.28	7.91		

the assumption that the "endogenous" D:N of the control period prevails during the experimental period, and the further assumption in the present instance that all of the nitrogen absorbed was excreted in the urine during the period for which the calculation is made. To obtain the extra sugar put out in the urine of the experimental period, the amount of nitrogen ingested is subtracted from the total nitrogen excreted; the remainder is multiplied by the D:N value for the control period; the product is deducted from the total reducing sugar excreted, and the remainder is taken as recovered dextrose.

Dog 34.—The preliminary regimen for this dog was similar to that employed in the first case described. On the morning of the 4th phlorhizin day the dog received by stomach tube 30 gm. of dextrose in 250 cc. of warm water. The data of this experiment are shown in Table II. Here, where no urea is given, the hourly rate of excretion of sugar is maintained above the control level for a considerably longer period (about 10 hours) than in the case of Dog 36. In Dog 34 we have, also, a coincident hyperglycemia of about the same order of magnitude as in Dog 36; but here, at the end of 6 hours, the blood sugar, while falling, is still well above its initial value.

In this experiment the "sparing" effect of dextrose, which is not itself burned, on the protein metabolism—a phenomenon to which Ringer (6) has called attention— is well shown in the diminished hourly excretion of nitrogen.

Of the dextrose given in this case, 89 per cent was recovered in the urine.

Dog 33.—The data of this case (Table III) represent our first experiment in which a mixture of dextrose and urea was fed. Here blood analyses were not made. Also, the experiment was somewhat complicated by the fact that the dog vomited several times, beginning about 4 hours after ingestion of the dextrose-urea mixture. The experiment is presented since the time relations of the excretion in the urine of sugar and nitrogen are practically identical with the results in Dog 36, corroborating a nitrogen lag relative to the excretion of sugar.

It may be of some significance, as bearing upon the question of possible differences in rate of absorption of the dextrose and urea, that the vomits obtained from the dog showed no change in the D:N ratio as compared with the mixture given.

Of the dextrose presumably absorbed, 86 per cent was recovered in the urine of the subsequent 24 hours. The calculation was

TABLE III.

Dog 33. Female. Weight, 11.5 kilos. Last feeding, Mar. 3. Beginning Mar. 6, 0.5 gm. of phlorhizin in olive oil was injected subcutaneously at 8.00 a.m. and 8.00 p.m. daily.

Date.	Urine.							Remarks.
	Time of collection.	Volume.	Total N.		Total sugar.		D:N	
		cc.	N per hr.	gm.	sugar per hr.	gm.		
Mar. 9	10.20 a.m.							Urine from 8.00 a.m.
	to							to 10.20 a.m., Mar.
" 10	8.00 a.m.	868	0.31	6.75	1.02	22.23	3.29	9, lost.

At 9.40 a.m., Mar. 10, given by stomach tube a mixture of 20 gm. of dextrose and 11.8 gm. of urea (5.5 gm. of nitrogen) in 200 cc. of warm water. In this mixture D : N = 3.6.

Date.	Time of collection.	Volume.	N per hr.	Total N.	sugar per hr.	Total sugar.	D:N	Remarks.
Mar. 10	9.30 a.m.	56	0.30	0.45	1.02	1.53	3.40	
	10.50 "	91	0.58	0.78	2.86	3.81	4.88	
	11.35 "	81	0.93	0.70	4.61	3.46	4.94	
	12.45 p.m.	138	1.16	1.35	4.93	5.75	4.26	
	1.45 "	86	0.92	0.92	2.84	2.84	3.09	Dog vomited at 1.50 p.m.; collected separately.*
	2.40 "	45	0.77	0.71	1.79	1.64	2.31	Dog vomited at 2.10 p.m.; collected separately.*
	4.05 "	52	0.62	0.88	1.20	1.70	1.93	Dog vomited at 4.40 p.m.; collected separately.*
	7.10 "	92	0.45	1.40	1.11	3.42	2.44	
	8.00 "	32	0.50	0.42	1.32	1.10	2.64	
	9.30 "	52	0.34	0.51	1.00	1.50	2.94	
	10.25 "	43	0.39	0.36	1.16	1.06	2.94	
" 11	12.00 a.m.	81	0.35	0.56	1.03	1.63	2.91	
	1.35 "	78	0.32	0.50	0.97	1.53	3.06	
	2.25 "	58	0.41	0.34	1.16	0.97	2.85	
	3.30 "	65	0.37	0.40	1.05	1.14	2.85	
	5.00 "	80	0.27	0.41	0.92	1.38	3.37	
	6.40 "	76	0.23	0.39	0.85	1.41	3.61	Dog failing rapidly.
	8.10 "	90	0.19	0.29	0.71	1.06	3.65	Phlorhizin omitted.†
Total		1,296		11.37		36.93	3.25	

* The vomits were analyzed separately for total nitrogen and dextrose: Vomit at 1.50 p.m.: Volume, 185 cc.; totalN, 0.38 gm.; total sugar, 1.36 gm.; D:N = 3.58.

made as in the case of Dog 36, the amounts of dextrose and urea absorbed being ascertained by subtracting from the weights ingested the amounts recovered in the vomits.

DISCUSSION.

In the dogs of our experiments we have demonstrated not only a hyperglycemia which was maintained for 6 hours or longer, but also the quantitative excretion, within accepted limits,[4] of the dextrose fed. *Despite a concentration of sugar within the phlorhizinized organism well above the normal level no sugar was burned.* It is difficult to agree with Lusk that the failure to burn sugar under these conditions may be due to acidosis. The acidosis of phlorhizin diabetes in dogs is frequently not as marked as the acidosis of diabetes mellitus in man where a partial sugar-burning capacity may be retained. Benedict and Osterberg (11) have pointed out that simple meat feeding to phlorhizinized dogs practically abolishes the acetone bodies even when none of the sugar formed is burned. Further, the acidosis in starvation is not attended by a commensurate failure to burn sugar. We take the view, rather, contrary to Allen, that phlorhizin *does* produce an intrinsic impairment of utilization of sugar by the tissues. Not only does phlorhizin affect the kidney tissue, but all other metabolizing tissues as well, and probably in much the same manner. Lusk (7) has shown that there are, apparently, three independent mechanisms within the cell for the combustion of amino-acids, fats, and sugar, respectively. We would advance the tentative

[4] Small discrepancies are to be attributed to the method of calculation, which involves the assumption of a constant urinary D:N throughout both the control and experimental periods. Ringer (6) has pointed out that, on a dynamogenetic basis, the actual burning of such small amounts of sugar cannot account for the degree to which protein combustion is spared.

Vomit at 2.10 p.m.: Volume, 210 cc.; total N, 0.39 gm.; total sugar, 1.39 gm.; D:N = 3.56.

Vomit at 4.40 p.m.: Volume, 185 cc.; total N, 0.13 gm.; total sugar, 0.22 gm.; D:N = 1.69.

† The condition of the dog rapidly became very bad. On Mar. 13 the animal was in such an apparently hopeless state that it was killed.

hypothesis that phlorhizin forms a relatively stable union with the "carbohydrate receptors" of the cells, with the result that sugar molecules can no longer enter into the hypothetical combination prerequisite to their combustion. We conceive that such a combination between phlorhizin and the carbohydrate receptors of kidney cells may account for their failure to retain sugar or to reabsorb it from urine. The phlorhizin-cell union is presumably considerably more stable than the corresponding dextrose combination. According to this view, the sugar starvation in phlorhizin diabetes is analogous to the oxygen starvation in carbon monoxide poisoning. It may be pointed out that phlorhizin is a glucoside and possibly has quite a similar grouping for a part of the molecule as compared to dextrose. Allen (2) has called attention to the fact that sucrose, another glucoside, when given subcutaneously causes the appearance in the urine of reducing material.

A second consideration of interest in our results is the time relations in the excretion of sugar and urea following the ingestion of these in a mixture. Our curves are strikingly similar to those found by Reilly, Nolan, and Lusk (12) after feeding meat to a phlorhizinized dog, and by Janney (13) after feeding various proteins to phlorhizinized dogs. Janney pointed out that the curves of nitrogen excretion in his experiments were not essentially different from results reported by various authors for normal dogs. Janney's findings, and those of Reilly, Nolan, and Lusk have been widely interpreted as indicating a more rapid production of sugar than urea from amino-acids. Janney, while apparently favoring this view, suggested the possibility that " . . . . the greater permeability of the phlorhizinized kidney for glucose may be the true explanation of this phenomenon." Our observations apparently substantiate the latter explanation.

CONCLUSION.

Dextrose ingested in sufficient amount to induce protracted hyperglycemia in phlorhizinized dogs is recovered quantitatively in the urine. The view is suggested that phlorhizin not only affects the permeability of the kidney tissue to blood sugar, but produces an intrinsic impairment of the sugar-burning mechanism.

When a mixture of dextrose and urea is ingested by a phlorhizinized dog, the sugar is excreted in the urine more rapidly than the urea. The phenomenon is similar to that observed when meat or proteins are fed to phlorhizinized dogs, and discredits the interpretation of a more rapid production of sugar than urea in amino-acid metabolism.

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SUGAR ELIMINATION AFTER THE SUBCUTANEOUS INJECTION OF GLUCOSE IN THE DOG.

INCLUDING A DISCUSSION OF THE PAPER ON OBSERVATIONS ON CARBOHYDRATES BY FOLIN AND BERGLUND.

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On the basis of a qualitative test for reducing sugar in the urine, it has been commonly assumed that the tolerance for subcutaneously injected glucose in the dog is about 5 gm. of glucose per kilo of body weight of the animal. Using a similar basis for detecting sugar excretion, Woodyatt (1) and collaborators have reported that sugar may be injected intravenously into the dog up to the rate of 0.85 gm. per kilo of body weight of the animal per hour before any of the injected glucose appears in the urine.

In the present work we have studied the tolerance of the dog for subcutaneously injected glucose on the basis of the actual sugar elimination following such injections as compared with control periods without sugar injections. The animals used were placed in metabolism cages upon a constant weighed diet and were catheterized 3 and 24 hours after feeding. Where glucose was given it was injected in 60 per cent solution into the neck or along the back or sides of the animal immediately after feeding. The sugar solutions were sterilized by boiling and injected with aseptic precautions. Only slight infections occasionally developed after the largest injections. The glucose used was either Kahlbaum's brown label product or Merck's "highest purity" dextrose. All the sugar samples employed were analyzed by Allihn's method and by the polariscope, and gave figures for 100 per cent glucose by these methods. Sugar was determined before and after treatment with yeast, by the acetone-picric acid method after clearing with bone-black (2), glucose being added as a check

on the activity of the yeast in every instance. The loss in reducing power of the urine, following treatment with yeast, is reported in the tables as "fermentable sugar." We also studied the effect of preliminary hydrolysis (boiling for 20 minutes with an equal volume of N hydrochloric acid), on the reducing power of the urine, and in the tables have indicated the increase due to such hydrolysis in a separate column. We do not regard these figures as indicating the true polysaccharide content of normal urine, as they may well include non-carbohydrate material. They should serve, however, to show whether any of the injected glucose is eliminated in the form of di- or polysaccharide.

Quite extended experiments with three animals were carried out, with very similar results in each case. In Tables I and II are detailed illustrative experiments with two animals.

We shall confine our discussion of the experiments chiefly to Table I, since the main points are brought out clearly in this table, and are essentially corroborated by the results in Table II.

It will be noted that following a subcutaneous injection of only 0.4 gm. of glucose per kilo of body weight, there is a definite increase in the total sugar output (March 31 and April 2, Table I). The excess sugar output is not confined to the first 3 hours after the injection, but apparently extends over most of the day of the injection. There is no definite influence of the injection on the non-fermentable sugar or on the polysaccharide content of the urine.

It seems indeed remarkable that such a small quantity of sugar (a total of 5.8 gm.) injected into a dog of over 14 kilos of weight should result in a definitely detectable loss of sugar through the kidneys, yet the results of both experiments are perfectly clear-cut. It should be noted that this finding was made where the injection was given simultaneously with feeding, and that we have failed to obtain any increase in a few experiments similar to this, which were carried out on a fasting animal. These results are quite in line with the studies reported on sugar tolerance for human beings by Benedict, Osterberg, and Neuwirth (3).

With the injection of 0.6 gm. of glucose per kilo of body weight (April 4, Table I), there is a more marked elimination of sugar in the urine, and the increase is wholly in the fermentable fraction. With the larger quantities of glucose there is a definite failure

to return to the normal urinary sugar level within 24 hours, the increase extending well into the 2nd day. This becomes especially marked following the injection of 4 gm. of glucose per kilo of body weight (April 20, Table I), when the urinary sugar remains above the normal level on April 21. A similar result is to be found in Table II (March 14 and 15). This "lag" in the excretion of the glucose is certainly difficult of explanation. It suggests that a portion of the sugar is changed in the organism to some form which is non-utilizable, and which is slowly eliminated from the body. That such changes may and do occur with pure glucose given subcutaneously is clearly shown in the experiments where 6, 7, and 8 gm. of glucose per kilo of body weight are injected.

The normal fluctuation in the yeast-resisting and the hydrolyzable sugar are too great to warrant any definite conclusion concerning the effect of the smaller quantities of glucose on the output of these sugars. With the larger quantities of glucose (April 23 and 25, Table I; March 16 and 19, Table II), where 6, 7, and 8 gm. per kilo of body weight of glucose were employed, there is a definite and unmistakable rise in every form of sugar in the urine, which extends at least into the 2nd day after the injection. Fermentable, non-fermentable, and di- or polysaccharide sugar are all markedly increased.

We believe that an increase in non-glucose sugar in the urine has never before been reported following the injection of pure glucose. Yet we feel that such a result should not be regarded as wholly unexpected. With these larger doses of sugar given subcutaneously the organism is suddenly confronted with the necessity of handling a large amount of glucose in a short space of time. We know that polymerization of glucose must occur in glycogen formation, and it would not be surprising if detectable quantities of intermediate products should escape into the urine. We feel that an investigation of the non-glucose sugars in the urine following large injections of glucose might throw interesting light upon the intermediate metabolism of glucose. Our preliminary experiments in this connection have shown that most (60 to 75 per cent) of the substance found after glucose injection which yields the increased reduction upon boiling with acid is destroyed by preliminary treatment with yeast. Hence we cannot be dealing here with conjugated glycuronic acid.

TABLE I

Showing the effect of subcutaneously injected glucose on the urinary sugar output. The sugar was injected in 60 per cent solution immediately after feeding

Female dog, weight 14 6 kilos Fed daily the following diet at 9 30 a m

	gm
Cracker meal	200
Meat	40
Evaporated milk	90
Bone ash	50

Date	Urine: Volume	Urine: Sugar before fermentation			Urine: Fermentable sugar	Urine: Non-fermentable sugar	Urine: Increase in total sugar due to hydrolysis	Remarks
1921	*cc*	*per cent*	*mg*	*mg per kg*	*mg*	*mg*	*mg*	
Mar 29								
9 30 a m to 12 30 p m	34	0 16	53	17 7	27	25	35	No glucose injected
12 30 p m to 9 30 a m	365	0 072	262	12 5	80	182	183	
Total for 24 hrs	399		315	13 2	107	207	218	
Mar. 30								
9 30 a m to 12 30 p m	56	0 091	51	17 0	25	25	28	No glucose injected
12 30 p m to 9 30 a m	380	0 066	251	11 9	83	167	141	
Total for 24 hrs	436		302	12 6	108	192	169	

Mar. 31								
9.30 a.m. to 12.30 p.m......	26	0.25	65	21.7	36	28	25	Injected 5.8 gm. glucose (0.4 gm. per kilo body weight).
12.30 p.m. to 9.30 a.m......	460	0.065	299	14.2	147	152	207	
Total for 24 hrs............	486		364	15.1	183	180	232	
Apr. 1								
9.30 a.m. to 12.30 p.m......	34	0.15	51	17.0	30	21	35	No glucose injected.
12.30 p.m. to 9.30 a.m......	450	0.063	283	13.5	139	144	176	
Total for 24 hrs............	484		334	13.9	169	165	211	
Apr. 2								
9.30 a.m. to 12.30 p.m......	30	0.21	64	21.3	35	29	39	Injected 5.8 gm. glucose (0.4 gm. per kilo body weight).
12.30 p.m. to 9.30 a.m......	380	0.077	292	13.9			145	
Total for 24 hrs............	410		356	14.9			184	
Apr. 3								
Total for 24 hrs............	530	0.057	302	12.6	95	196	247	No glucose injected.
Apr. 4								
9.30 a.m. to 12.30 p.m......	24	0.26	63	21.0	32	31	35	Injected 8.7 gm. glucose (0.6 gm. per kilo body weight).
12.30 p.m. to 9.30 a.m.	290	0.127	368	17.5	203	165	212	
Total for 24 hrs............	314		431	18.0	235	196	247	
Apr. 5								
9.30 a.m. to 12.30 p.m......	76	0.074	56	18.7	30	26	29	No glucose injected.
12.30 p.m. to 9.30 a.m.. ..	450	0.071	319	15.2	121	198	207	
Total for 24 hrs............	526		375	15.6	151	224	236	

TABLE I—*Concluded.*

Date.	Urine. Volume.	Sugar before fermentation.			Fermentable sugar.	Non-fermentable sugar.	Increase in total sugar due to hydrolysis.	Remarks.
1921	*cc.*	*per cent*	*mg.*	*mg per hr.*	*mg.*	*mg*	*mg*	
Apr. 6								
Total for 24 hrs.	474		416	17.4	214	199	173	Injected 11.6 gm. glucose (0.8 gm. per kilo body weight).
Apr. 7								
Total for 24 hrs.	480		326	13.6	103	223	177	No glucose injected.
Apr. 8								
9.30 a.m. to 12.30 p.m.	22	0.30	66	22.0	38	28	44	Injected 14.6 gm. glucose (1 gm. per kilo body weight).
12.30 p.m. to 9.30 a.m.	640	0.063	403	19.2	211	192	224	
Total for 24 hrs.	662		469	19.6	249	220	268	
Apr. 9								
Total for 24 hrs.	450		376	15.7	187	189	204	No glucose injected.
Apr. 11								
Total for 24 hrs.	598		478	20.0	252	226	314	Injected 21.7 gm. glucose (1.5 gm. per kilo body weight).
Apr. 12								
Total for 24 hrs.	498		365	15.2	150	214	185	No glucose injected.

Apr. 18								
Total for 24 hrs............	425		571	23.8	320	251	173	Injected 43.5 gm. glucose (3 gm. per kilo body weight).
Apr. 20								
Total for 24 hrs............	225		685	28.5	403	282	197	Injected 58.4 gm. glucose (4 gm. per kilo body weight).
Apr. 21								
Total for 24 hrs............	985		492	20.5	229	263	269	No glucose injected.
Apr. 22								
9.30 a.m. to 12.30 p.m......	20	0.50	100	33.3	65	35.2	48	Injected 73 gm. glucose (5 gm. per kilo body weight).
12.30 p.m. to 9.30 a.m......	950	0.060	570	27.1	351	218.0	237	
Total for 24 hrs............	970		670	28.0	416	253.2	285	
Apr. 23								
Total for 24 hrs.	1,106		701	29.2	406	295	385	Injected 87.6 gm. glucose (6 gm. per kilo body weight).
Apr. 24								
Total for 24 hrs......	1,090	0.035	378	16.4	181	196	134	No glucose injected.
Apr. 25								
9.30 a.m. to 12.30 p.m......	10	1.21*	121	40.0	84	37	96	Injected 102 gm. glucose (7 gm. per kilo body weight.
12.30 p.m. to 9.30 a.m.... .	1,380	0.048	662	34.9	331	331	553	
Total for 24 hrs..	1,390		783	35.6	415	368	649	
Apr. 26								
Total for 24 hrs...........	1,716		664	27.7	331	332	231	No sugar injected.

* Blood drawn at 11.00 a.m. showed 0.12 per cent of sugar.

TABLE II.

Showing the effect of subcutaneously injected glucose on the urinary sugar output. The sugar was injected immediately after feeding.

Female dog, weight 12.3 kilos. Fed daily the following diet at 10.00 a.m.

	gm.
Cracker meal	175
Meat	30
Evaporated milk	70
Bone ash	60

Date.	Urine. Volume.	Sugar before fermentation.			Fermentable sugar.	Non-fermentable sugar.	Increase in total sugar due to hydrolsyis.	Remarks.
1921	*cc.*	*per cent*	*mg.*	*mg. per hr.*	*mg.*	*mg.*	*mg.*	
Feb. 20								
Total for 24 hrs.	410		283	11.8	139	143	98	No glucose injected.
Feb. 23								
Total for 24 hrs.	317		271	11.3	122	149	127	No glucose injected.
Feb. 24								
10.00 a.m. to 1.00 p.m.	28	0.13	37	12.4	16	21	23	No glucose injected.
1.00 p.m. to 10.00 a.m.			252	12.0	109	142	87	
Total for 24 hrs.			289	12.1	125	163	110	
Feb. 25								
10.00 a.m. to 1.00 p.m.	14	0.32	45	15.2	27	18	18	Injected 10 gm. glucose (0.81 gm. per kilo body weight).
1.00 p.m. to 10.00 a.m.	182	0.15	287	13.7	143	143	126	
Total for 24 hrs.	196		332	13.9	170	161	144	

Feb. 26								Injected glucose as on Feb. 25.
Total for 24 hrs.	444		312	13.0	152	160	134	
Feb. 27								
Total for 24 hrs.	530		307	12.8	121	185	164	No glucose injected.
Feb. 28								
Total for 24 hrs.	291		288	12.0	116	171	106	No glucose injected.
Mar. 2								
10.00 a.m. to 1.00 p.m.	14	0.36	50	16.8	24	26	32	Injected 15 gm. glucose (1.2 gm. per kilo body weight).
1.00 p.m. to 10.00 a.m.	180	0.16	293	14.0	124	169	121	
Total for 24 hrs.	194		343	14.3	148	195	153	
Mar. 14								Injected 55 gm. glucose (4.5 gm. per kilo body weight).
Total for 24 hrs.	330		476	19.8	190	285	243	
Mar. 15								
Total for 24 hrs.	530		345	14.4	176	169	163	No glucose injected.
Mar. 16								
10.00 a.m. to 1.00 p.m.	10	0.94	94	31.3	48	46	53	Injected 98 gm. glucose (8 gm. per kilo body weight).
1.00 p.m. to 10.00 a.m.	450	0.10	450	21.4	184	265	288	
Total for 24 hrs.	460		544	22.7	232	311	341	
Mar. 17								
Total for 24 hrs.	630		402	16.8	202	200	246	No glucose injected.
Mar. 18								
Total for 24 hrs.	360		344	14.3	177	167	246	No glucose injected.
Mar. 19								
10.00 a.m. to 1.00 p.m.	10	0.97	97	32.3	62	35	75	Injected glucose as on Mar. 16 (8 gm. per kilo body weight).
1.00 p.m. to 10.00 a.m.	830	0.05	456	21.7	207	249	349	
Total for 24 hrs.	840		543	23.1	269	284	424	

It is interesting to note that following an injection of 7 gm. of glucose per kilo of body weight (Table I, April 25), a sample of blood showed a sugar content of only 0.12 per cent (by the picrate method) for a period when the urine sugar contained 1.21 per cent of reducing sugar. The blood was taken exactly in the middle of the 3 hour period. While the exact sugar content of the urine for the instant that the blood was drawn cannot be known, it is certain from numerous experiments on the general curve of sugar excretion that the urine sugar in this case was many times above normal when the blood sugar was 0.12 per cent. This finding seems to us again to indicate a definite excretion of some waste sugar rather than the simple filtering past the kidney of an excess of glucose. We have frequently found a figure over 0.1 per cent for the blood sugar of the dog following simple handling of the animal, and when the urine contained less than one-third of the 1.2 per cent found in this instance.

When we remember the possible isomerism of the hexose sugars, and the fact that pure glucose solutions represent an equilibrium between two varieties of *d*-glucose, it seems quite possible to understand how the introduction of a mixture of α and β glucose should be followed by excretion of pure glucose, which may be really a waste product. In view of the well known specific character of cellular activity, it is hardly likely that the two varieties of glucose are equally utilizable by the body, or that they normally circulate in the blood in the same relative concentrations in which they exist in pure aqueous solution. Following the injection of considerable quantities of glucose solution, the two sugars therein contained presumably enter the blood stream in unusual relative, if not absolute, quantities. The tissues may then pick out a portion which is really a foreign sugar, and which may later be given off into the blood stream and reach the kidney before it undergoes some necessary transformation in the liver or other specific tissue. It is only along the line of some such view as this that we can even partially interpret such results as we here report, following the subcutaneous introduction of what analyzes as pure glucose. Not only the prolonged excretion of sugar following a single injection of glucose may be interpreted along this line, but we may also understand how, following injection of pure glucose, a high concentration of sugar in the urine (1.2 per cent)

may occur when the blood sugar is very slightly above normal (0.12 per cent), as we report in connection with Table I (*cf.* the suggestive discussion by Woodyatt (1); also the recent work of Winter and Smith (4).

The work above presented was carried out about 2 years ago. Publication was delayed because we hoped to elucidate the nature of the urinary sugars following introduction of the larger quantities of glucose. Progress in this work is difficult, and we have been frequently interrupted. In the meantime a long paper by Folin and Berglund (5) has appeared, in which results following carbohydrate administration are reported, which are essentially contrary to many earlier findings, and in which vigorous exception is taken to some of the conclusions reached by Benedict, Osterberg, and Neuwirth (3) in their work on the urinary sugar excretion. We have, therefore, felt it desirable to publish the above experiments with pure glucose without further delay, and to include a discussion of some of the points raised by Folin and Berglund in their somewhat extended presentation of closely related questions.

The general method adopted by Folin and Berglund in their study of carbohydrate utilization was essentially that of following the blood sugar and urine sugar after the ingestion of very large amounts of pure carbohydrate material. The experimental subjects were young men. Of the twenty-three experiments reported in detail, fifteen, or 65 per cent, were carried out upon one subject, H. B-d. Of the remaining eight experiments, three are reported as having been carried out upon two subjects abnormal in some relation to carbohydrate tolerance. Thus subject H. B-d. was employed in 75 per cent of all of the experiments reported by Folin and Berglund which purported to be carried out upon normal human individuals. Four different subjects served for the remaining five experiments. It is stated, however, that numerous other experiments were carried out similar to those reported.

The objects, and in general the method of study of Folin and Berglund did not differ materially from those of previous investigators along the same line. The experiments reported for subject H. B-d. were quite extended, but the remaining experiments, being so few in number, covered the ground studied rather less thoroughly than had already been done by previous investiga-

tors. The conclusions reached as a result of the study are sweeping in nature, and differ very sharply in most points from the conclusions reached by those who had studied similar questions before.

The first point made by Folin and Berglund as a result of their work is that the giving of 200 gm. of glucose on an empty stomach in no normal case yielded "a blood sugar high enough to give the alimentary glycosuria which comes when the glucose threshold is reached." And further "the giving of 200 gm. of pure glucose not only failed to produce the glycosuria which accompanies the blood sugar threshold, but the tables show that the hyperglycemias which we did obtain are wholly without effect on those lower levels of sugar excretion comprised in the term glycuresis."

Conclusions contrary to these have been reached by many of the earlier investigators. Some of this work is cited by Folin and Berglund. Thus they mention the work of Jacobsen (6), who found alimentary glycosuria and high blood sugars (160 to 170 mg.) in 57 per cent of the fourteen subjects (presumably normal) to whom he gave only 100 gm. of glucose on an empty stomach. Folin and Berglund suggest the rather ingenious explanation of this finding, that the drop method of blood collection employed by Jacobsen hurt the subjects more than does venous puncture (employed by Folin and Berglund) and caused a "superimposed psychical hyperglycemia," which may explain Jacobsen's results. If we are to accept such an explanation, we shall have to regard all experiments where blood is drawn at all as of very doubtful value. If the difference between the pain due to finger puncture and the pain due to venous puncture is (as Folin and Berglund would suggest) of such fundamental significance in relation to hyperglycemia and to sugar excretion, any method of study of the problem involving the slightest discomfort or even uneasiness of the subject would be valueless. Venous puncture, and especially the sight of blood, might well be too intolerable for subjects to endure without an outpouring of sugar into the blood and the urine.[1] Goto and Kuno (7), however, obtained quite similar

[1] Since this paper was written Foster (Foster, G. L., *J. Biol. Chem.*, 1923, lv, 291) has presented a study of the questions involved in the comparative sugar values for venous and finger blood. Foster's results in this connection seem to show conclusively that emotional elements need not be invoked to explain the sugar content of finger blood.

results for the urine sugar to those of Jacobsen without employing finger puncture. These investigators studied the effect of 100 gm. of glucose upon the blood sugar and glycosuria in 53 Japanese subjects, and employed venous puncture for collecting the blood. Twenty-two, or 41 per cent, of the subjects showed glycosuria following *100* gm. of glucose, and thirty-three, or 60 per cent, showed some increased sugar excretion. Myers (8), employing venous puncture for blood collection, cites a presumably normal individual who showed glycosuria for a short period in a test with 75 gm. of glucose. Taylor and Hulton (9) studied the question of glycosuria after ingestion of large quantities of pure glucose, but examined the urines for sugar only in the sample for 24 hours after the sugar ingestion. Folin and Berglund (5), in referring to this work, state "we would have the reader recall that A. E. Taylor some years ago also met with uniform failure in attempting to produce alimentary glycosuria in normal persons (students) by feeding pure glucose. Taylor evidently was not aware of the transient character of the hyperglycemia produced by sugar, for he examined only 24 hour urines."

We must correct somewhat Folin and Berglund's summary of A. E. Taylor's work in this connection by pointing out that in six out of twenty-six students to whom he gave 200 gm. of glucose, alimentary glycosuria was found in examination of the 24 hour urine following such sugar ingestion. Of the nine students who received 300 gm. of glucose, and who had failed to show sugar in the urine on 200 gm. of glucose, three, or 33 per cent, showed sugar following the larger quantity. The same percentage held for the "resistant" students of this group who received 400 gm. of glucose while, of five individuals who received 500 gm. of glucose, only one showed reducing sugar in the following 24 hour urine. Since Taylor found 23 per cent of his subjects showed glycosuria upon 200 gm. of glucose in the 24 hour urine, and larger percentages with 300 and 400 gm. of sugar, it is obviously a mistake to say that his efforts in this connection resulted in "uniform failure." In view of the transitory nature of the glycosuria following glucose ingestion (Myers, 8), there is every reason to believe that Taylor would have found glycosuria in two or three times as many of his subjects had he examined their urine during frequent intervals after ingestion of the sugar.

In duplicate experiments on two men, Benedict, Osterberg, and Neuwirth (3) showed a perfectly definite increase in the sugar output following, in one case 40 gm. of glucose and in another 60 gm. of glucose[2] given on an empty stomach.

In this partial comparison of previous findings in regard to the urinary sugar output following glucose ingestion, one is at once impressed with the sharply contrasted conclusions drawn by the earlier investigators (except Taylor), as compared with those reached by Folin and Berglund.

Jacobsen found glycosuria in 57 per cent of his subjects following ingestion of 100 gm. of glucose. Goto and Kuno found it in 41 per cent of their subjects following ingestion of the same quantity of glucose. Myers reported glycosuria in a single normal subject following ingestion of 75 gm. of pure glucose. Benedict, Osterberg, and Neuwirth demonstrated increased sugar excretion following ingestion of 40 and of 60 gm. of pure glucose in individuals who have remained well and free from diabetes for 5 years since those experiments.

Folin and Berglund, however, report that normal individuals show no increase in the urinary sugar output following the ingestion of 200 gm. of pure glucose. We shall examine their data for this conclusion in some detail later. For the present it is sufficient to take for granted the general proposition that several other investigators report *readily demonstrable* increases in the urinary sugar following the ingestion of 100 gm. of glucose, while Folin and Berglund are unable to find such increases in any normal individual after giving 200 gm. of glucose. We feel sure that these conflicting results cannot be reconciled on a basis of impure glucose employed. Neither do we feel that the explanation lies in the technique employed by Folin and Berglund, or in the fact that these investigators alone used normal individuals for such experiments.

In attempting to reconcile the conflicting views noted, the first point which deserves consideration is the fact that Folin and Berglund have apparently not duplicated the essential conditions which were adopted by those previous experimenters whose con-

[2] The glucose employed in all the experiments of Benedict, Osterberg, and Neuwirth was either Kahlbaum's or Merck's best grade, and was perfectly pure according to our analytical figures.

clusions they so vigorously dispute. With one single exception, in which 30 gm. of glucose were employed, Folin and Berglund used 200 gm. or over as their fixed dose of glucose. The work which we have cited as leading to conclusions contrary to those of Folin and Berglund was founded on the use of 40, 60, 75, and in most cases 100 gm. of glucose, or one-half or less of the quantity of glucose employed by Folin and Berglund. Possibly many would, at first thought, assume that Folin and Berglund strengthened their argument by doubling the quantity of glucose used. But such a point of view cannot be adopted to refute positive evidence founded on the use of a different amount of glucose. Quantities of 40 to 100 gm. of glucose might perhaps be regarded as reasonably near physiological doses of the sugar. When, however, 200 gm. of glucose are employed, we are inclined to raise the question as to whether the absorptive and eliminative processes may be regarded as necessarily normal. It appears that the introdudtion of a liter of 20 per cent glucose, or a half liter of 40 per cent glucose into the stomach at one time may be definitely abnormal for the organism, and that neither normal absorption nor elimination need necessarily be expected to follow such excessive doses of strongly hypertonic solution. As a matter of fact there is evidence already in the literature that sugar may very definitely decrease the general secretory activity of the kidney. Thus the antidiuretic action of large doses of sugar is too well known to need comment beyond mentioning the very long, and wholly unusual period required for elimination of the water which accompanies the sugar in such experiments (10). Mendel and Rose (11) mention that following feeding of rabbits with pure sugars (glucose and cane-sugar) "frequently the kidney excretion was practically stopped after giving the sugar for two or three days, and the animals died with symptoms of uremic poisoning."

We may also mention in this connection an experiment carried out some years ago by us in which a dog received 17 gm. of glucose per kilo of body weight in 70 per cent solution subcutaneously. The animal died at the end of 17 hours after the injection. The urine secreted during this period, amounting to 31 cc., was nearly as colorless as water. It yielded no qualitative test for sugar, chlorides, or sulfates. We cite the experiment because it shows

an extreme effect of sugar on the kidney, and therefore serves to call attention to a possible effect of smaller doses.

What we consider excellent evidence of the inhibiting action upon the kidney activity of the very quantity of sugar Folin and Berglund employed is contained in a paper by these same investigators, detailing their study on the excretion of amino-acids (12). In Table VIII of that paper the subject (D-n.) was fasting and took 200 gm. of glucose. At the end of an hour and a half after taking the glucose, the amino-acid nitrogen in the urine had decreased nearly 50 per cent and the urea nitrogen had dropped 60 mg. per hour. And this, in spite of the fact that the amino-acid nitrogen in the blood was unchanged for at least the first 50 minutes, while the only change in the urea nitrogen in the blood was a slight increase during the first 50 minutes with return to the previous level for the next 4 hours. The subsequent periods corroborate the view that the kidney activity is cut down at first markedly by the sugar, followed by a slow recovery. Folin and Berglund would explain the decrease in amino-acid and other nitrogen of the urine following the sugar as due to the sparing action of the sugar upon the nitrogen metabolism. This view seems hardly warranted, in view of the fact that the amino-acid nitrogen of the urine is largely (according to their interpretation) a mechanical loss through the kidney controlled by the concentration in the blood, and the additional fact that the urea nitrogen in the blood did not fall while that in the urine decreased. At any rate our explanation seems quite as plausible as the view taken by Folin and Berglund.

We do not feel inclined to urge depressant action of the glucose on kidney function as the probable explanation of the discrepancies between the results of Folin and Berglund and others, including ourselves, but suggest it merely as a possibility. Perhaps overemphasis of the emotional factor in some experiments contributed to the findings.[3]

[3] Apparently Folin and Berglund have adopted emotional disturbance as an explanation of hyperglycemia and glycuresis when this factor could not adequately explain the observed facts. Thus, in discussing Table XXI in their paper they state that the subject, Mr. S-g., fainted the second time the blood was drawn, and offer the emotional factor as an explanation of the high blood sugar and a urine sugar reaching 1.2 gm. per hour which followed ingestion of 200 gm. of glucose. But in the same table they show that the

As will develop later, we feel that Folin and Berglund have frequently failed to appreciate the real significance of their experiments, and, if so, this fact probably contributed to their reaching the conclusion which they did. In any event, we can find no reason to change our view that many normal individuals, under no nervous excitement, and without the drawing of any blood, will show increased sugar excretion, *i.e.* glycuresis, after the ingestion of 100 gm. or less of glucose. We have not maintained that all individuals show increased sugar after glucose ingestion upon an empty stomach. On the contrary, we previously stated (3): "In man there is apparently a real tolerance for glucose and for cane sugar, during fasting." As a result of the work of Folin and Berglund we are inclined to question this latter view, and tend to feel that there is no *absolute* tolerance for glucose in the human being. In support of this, we cite the experiment presented by Folin and Berglund (5) in Table XX. Here they report the only experiment with a small amount (30 gm.) of glucose on one whom they consider a thoroughly normal individual. The control sugar elimination amounted to 17 mg. per hour, which was maintained exactly during the first period of 18 minutes and the second period of 20 minutes after giving the sugar. During the third period (22 minutes duration) after giving the sugar, the urinary sugar represented 19 mg. per hour, and during the fourth period (16 minutes) 22 mg. per hour. During the fifth and last period recorded, the sugar elimination fell to 19 mg. per hour. Here is an unmistakable and typical curve showing the rise and fall of urinary sugar following the ingestion of 30 gm. of sugar, and wherein the greatest increase amounted to 29 per cent above the control level. It is, of course, not justifiable to draw conclusions from one experiment; nevertheless, the form of the sugar curve here is so typical as to warrant the inference that the glucose ingested was responsible for the rise in the sugar excreted. Folin and Berglund do not interpret this experiment as we do.

We may now consider the five experiments reported by Folin

same high urine sugar followed the evening meal, eaten several hours after blood and urine sugar had returned to normal. Assuming that this subject was not seriously upset emotionally by his accustomed dinner, we cannot feel that emotional disturbance was necessarily, or even probably, the cause of the glycuresis following the glucose ingestion.

and Berglund (5), in which they gave normal subjects 200 gm. (in one case 215 gm.) of glucose in from 500 to 1,000 cc. of water on an empty stomach, and determined the blood and urine sugar at intervals before and after the glucose ingestion. As a result of these, and similar unreported experiments, Folin and Berglund conclude that the results prove "*the concept comprised in the term glucose threshold to be not something only approximately true; the concept is absolutely correct, however uncertain the exact figures given for the threshold may be. Hyperglycemia definitely below the threshold does not normally produce the slightest leakage of glucose through the kidneys, and normally not a trace of absorbed and circulating glucose is lost.*"

Assuming for the moment that the figures of Folin and Berglund should not be differently interpreted, we cannot see that the failure to find any glucose threshold for normal men proves the concept of such a threshold to be correct. It would simply prove that no glucose threshold was found for human beings, in spite of the fact that plasma sugars up to 172 mg. were obtained, and would naturally lead to the question as to whether any glucose threshold exists for normal human beings.

The conclusion reached by Folin and Berglund that the normal kidney is absolutely efficient against the "*slightest leakage of glucose*" so that normally "*not a trace of absorbed and circulating glucose is lost*" seems to us of such prime importance as a starting point in all considerations of the behavior of sugar in the animal organism, involving as it does the corollary that normal urine never contains any glucose whatever, that we are led to examine quite closely the figures upon which this conclusion is based.

Let us then forget for a moment the interpretation placed upon their own figures in this connection by Folin and Berglund, and seek to examine the figures objectively to determine whether ingestion of the glucose has been followed by any increase whatever in the urinary sugar which can be properly credited to the ingested sugar.

On looking over Tables I to V, inclusive, of Folin and Berglund's (5), we note at once that in every instance there is a *definite general form* to the curve of the sugar elimination following the ingestion of the sugar. The general form of this curve is a *rise in the middle* and a *fall at both ends*. The form of the curve, taken with the

fact that in *no single one of the experiments* is the maximal figure for sugar elimination to be found either in the preliminary period, or at the close of the experimental period (about 6 hours total duration), offers convincing evidence that the experimental factor (glucose ingestion) was responsible for an increased urinary sugar excretion in these experiments. We, therefore, conclude that Folin and Berglund failed to interpret properly their experiments in this connection, and on the contrary drew a conclusion of far reaching moment which was contrary to the facts brought out by their figures. It may be urged that in any event the increases were too slight to have any significance. In relation to the statement italicized by Folin and Berglund in connection with this work, any increase, however small, must have significance. It can be easily shown, however, that the increases found were readily demonstrable and worthy of notice. The analytical method for sugar applied to the urine presumably had an accuracy of within 5 per cent of the quantity of sugar determined, and the urine volumes were large enough in every case to be collected within much smaller limits of accuracy. Yet we note that the percentage increases above the control period reached in Table I, 9 per cent; in Table II, 75 per cent; in Table III, 13 per cent; in Table IV, 28 per cent; and in Table V, 15 per cent. In milligrams, the increases above the control period ranged from 2 mg. per hour (Table I) to 15 mg. per hour (Table II). It is not clear why Folin and Berglund should head a table such as Table II "*Result:* Maximum subthreshold hyperglycemia but no glycuresis," when there was an increase in the sugar elimination reaching 75 per cent above (almost double) that of the control period.

We may cite conclusions drawn by Folin and Berglund themselves in another connection as basis for the view that the increment actually found in their five experiments with pure glucose should receive recognition. In their study of amino-acid distribution, Folin and Berglund (12) report an increase in amino-acid nitrogen in the plasma amounting to a maximum of 1.2 mg. per 100 cc. above the control period (from 5.2 to 6.4 mg. per 100 cc.), and in connection with this finding they state "there was obtained a definite, unmistakable increase in the amino-acid content of the blood." And further on, in connection with this experiment they state: "It will further be noted that the amino-acid excretion

with the *urine*" (a maximum rise of 2.6 mg. per hour) "confirms the values obtained for the blood."

We feel convinced that Folin and Berglund have repeatedly interpreted incorrectly the figures in their carbohydrate work, and as we deem an appreciation of this fact necessary to a proper estimate of the conclusions they reach, we shall here cite another discrepancy in the interpretation of their carbohydrate figures. Subject McC-n. was said to be a renal glycosuric and Table XIX (5) is cited to show that this subject exhibited glycuresis and glycosuria following 30 gm. of glucose. In this experiment the urine was collected in periods of less than ½ hour (which would best tend to reveal maximal figures). The sugar reached a maximal concentration of 0.16 per cent, and the total sugar recovered over that expected for the whole experiment was 39 mg., the total period of excess sugar excretion lasting about 1 hour and 6 minutes. For a period of 18 minutes a urine was secreted which contained 0.16 per cent sugar, and which was stated to give a positive qualitative test. Thus this experiment was interpreted (very properly) as showing glycuresis and glycosuria. In the experiment with glucose recorded in Table II the urine was collected in 1 or 2 hour periods, for 5 hours. In this experiment, a total of 79 mg. of sugar in excess of that expected was recovered and a concentration of sugar in the urine of 0.12 per cent was reached. Yet Folin and Berglund interpreted this sugar experiment as negative, while interpreting one in which there was one-half the absolute increase in sugar as positive.

We may now proceed to consider the evidence offered by Folin and Berglund as a basis for their conclusion that the glycuresis observed by Benedict, Osterberg, and Neuwirth (3), and by themselves, to follow every ordinary meal, represents simply the passage of foreign unutilizable carbohydrate contained in the food into the urine. Folin and Berglund's points in this connection require no very detailed discussion and for two reasons: (*a*), because their own experiments appear to prove at once the untenability of their conclusions; and, (*b*), because we feel that little additional discussion is necessary to show that, in this connection, Folin and Berglund have consistently misinterpreted the results of their experiments.

On page 235, Folin and Berglund (5) state: "The definite unmistakable glycuresis described by Benedict is obtained after every carbohydrate meal, but not after meals containing no carbohydrate (Table V)." If this statement is correct it must tend to discredit the view of glycuresis adopted by the present writer; and it is obviously a pivotal point in Folin's criticism of that position. We shall, therefore, consider the data offered by Folin and Berglund in Table V and see just how well the single experiment there reported supports the statement above quoted. Prior to the protein-fat meal the sugar output was 21 mg. per hour, and was falling after a 15 per cent rise following glucose ingestion. The sample of urine covering 3 hours and 45 minutes subsequent to the meal showed a sugar elimination for this period of 26 mg. per hour, or an increase of over 23 per cent for nearly 4 hours after eating the protein-fat meal. Opposite the figure showing the increase from 21 to 26 mg. per hour for nearly 4 hours after ingestion of the protein-fat meal, stands the legend "No glycuresis." Nevertheless, we should hardly expect a better vindication of our general view of glycuresis than is presented in the sustained definite increase in sugar excretion following a meal free from any carbohydrate. In our own work, Subjects E. O. and I. N. (3) showed increases in the 24 hour sugar output of 20 and 26 per cent, respectively, after changing from a high protein, carbohydrate-poor diet to an ordinary diet. To find a similar increase following a protein-fat meal as compared with fasting, extending for at least as long as 4 hours is indeed more than we should have expected. Assuming the validity of the data in this experiment, we believe that Folin and Berglund have here succeeded in proving the correctness of our view-point that the organism during digestion has no absolute tolerance for sugar; so that their own general position in this connection becomes quite untenable. It is not clear to us why investigators who so readily find an increase of 1 mg. of amino-acid nitrogen per 100 cc. of blood, and point to it as representing a 20 per cent rise, should fail to recognize a 23 per cent increase in the sugar output. But we note similar instances throughout the carbohydrate experiments of Folin and Berglund. Ingestion of maltose was followed by an increase in the sugar excretion reaching 25 per cent, which is recorded as "no glycuresis." Ingestion of 200 gm. of dextrin yielded the follow-

ing figures for reducing sugar in the urine: Control period 25 mg. of sugar per hour; first period after dextrin 27; second period 28; third period 31; fourth period 40; fifth period 27; sixth period 24; seventh period 25 mg. of sugar per hour. This typical curve, reaching a height 60 per cent above the control period, is interpreted by Folin and Berglund as showing no effect on the preformed sugar of the urine. 200 gm. of dextrin-starch showed the same form of curve, reaching a maximum 26 per cent above the control figure. The experiment is recorded as "Showing Absence of Glycuresis after 200 Gm. Pure Dextrin-Starch."

Nothing further is gained by analyzing in detail any more of the experiments recorded in this connection by Folin and Berglund. In some cases single experiments are reported in which the control sugar level is at least 50 per cent above the true fasting level for the subject as shown in the other experiments.

Experiments such as that of taking a liter of egg white at one dose do not especially interest us. A more nearly normal intake of food would seem preferable. One of us (E. O.) once took a liter of egg white and recovered about 50 per cent of the nitrogen in the feces (Wolf, 13). Bateman (14) reports an extended study of the indigestibility of raw egg white.

In referring to the glycuresis following gelatin, Folin and Berglund state: "It is difficult to tell what impurities may be present in commercial gelatin preparations." This may be true, but it is not difficult to determine whether any given sample of gelatin contains reducing sugar. We have examined several samples and failed to find any such sugar. In this connection we might mention the finding by Janney (15) that gelatin yields an exceptionally large quantity of glucose in the organism (65 per cent).

The experiment with 200 gm. of superheated levulose, so badly decomposed that it had lost 30 per cent of its reducing power, seems to us of very questionable value as evidence in regard to the origin of the sugar of normal urine. Less than half a gram of reducing sugar was recovered in the urine after ingestion of this strong cathartic.

We feel inclined, in addition, to raise the question as to whether subject H. B-d. is normal in his response to carbohydrate ingestion. Out of the fifteen or more experiments carried out on this one subject, it is noteworthy that in spite of the enormous quanti-

ties of carbohydrate ingested (up to 350 gm. of dextrin in one experiment), only in one single instance (after 200 gm. of glucose) was a hyperglycemia obtained. Special theories are advanced to explain the absence of hyperglycemia after 200 gm. of levulose, but no note is made of the absence of hyperglycemia after taking 100 gm. of galactose plus 100 gm. of glucose, though Folin and Berglund clearly recognize[4] that 100 gm. of glucose produce as great a hyperglycemia as does 200 gm. and that galactose is a poor glycogen former. The failure of 200 gm. of maltose to cause hyperglycemia in subject H.B-d. is also worthy of note. We should hesitate to conclude that such an individual is wholly normal. The indications would seem to be that he has slow absorption from the alimentary canal.[5]

Coming to consider another point, we believe that Folin and Berglund have, throughout their consideration of the origin of urinary sugar, made a fundamental assumption which is quite unwarranted by facts so far available. They appear to believe that if urine sugar is at all independent of blood sugar concentration, then none of the urine sugar can arise from the glucose in the blood. Woodyatt (1) presents a different viewpoint and we ourselves feel convinced that postulation of necessary connection between blood sugar concentration and excretion of urine sugar is not unavoidable, and is contrary to known facts. The more we have hunted for the elusive "glucose threshold," the more we feel that this is quite possibly wholly an artifact. We tend to adopt the view that the causes leading to glucose excretion by the kidney are usually the same as those leading to an increase in the blood sugar, but we question that the two latter phenomena need be always causally related. The low "threshold" at which glucose appears in the urine in large amounts in some cases of early diabetes, and the persistence of high blood sugar after cessation of glycosuria in diabetes would be in accord with our view. A difference in the state of the blood sugar, such as in the equilibrium existing between two varieties of sugar in the blood, might result in urine sugar excretion quite independently of the blood sugar concentration, but might lead at the same time to an increase in the blood sugar. One fact definitely in line with

[4] Folin and Berglund (3), p. 249.

[5] *Cf.* Foster.[1]

this view is the repeated finding that the *total* sugar eliminated is wholly unrelated to the maximum height, or the duration of the hyperglycemia, a fact which holds for no other known excretory function of the kidney. We may cite an example of this kind from the paper of Folin and Berglund. Their "renal glycosuric," subject McC-n. following ingestion of 30 gm. of glucose, gave a blood sugar of 139 mg., and eliminated extra urine sugar amounting to a total of 39 mg. The same subject, after ingestion of 200 gm. of glucose, gave a blood sugar of 113 mg., and eliminated extra sugar amounting to nearly a gram, though the blood sugar was practically normal throughout the entire period of this sugar elimination. Folin and Berglund would explain the continued elimination of sugar by the kidney after the blood sugar has reached normal, by assuming that the kidney is under a strain due to the increased blood sugar, and that the damage thus produced is not repaired at once, so that sugar elimination continues even after the blood sugar falls below normal. This explanation might seem adequate were it not that the "strain" on the kidney would seemingly have to be proportional to the total sugar in the organism including even unabsorbed glucose in the stomach, rather than to the height of the blood sugar. Thus, in subject Mc-C-n. the higher blood sugar which followed the 30 gm. of glucose would have had to produce less damage than the lower blood sugar which followed the 200 gm. of glucose. Such an assumption would, of course, be unreasonable. The actual findings are much more in accord with the view that the sugar elimination represents a period of adjustment, during which a portion of the newly arrived sugar is in a form which cannot be completely retained by the organism.

Before concluding, we should like to discuss briefly one further point raised by Folin and Berglund. These investigators noted that when 100 gm. of galactose were given to subject H. B-d., there was an extra sugar elimination of 5.6 gm., but that when the same quantity of galactose was given in a mixture with 100 gm. of glucose, the extra sugar eliminated amounted to less than one-tenth of that obtained with the galactose alone. From this observation Folin draws the very interesting tentative conclusion that "the extent to which galactose is retained and utilized by the human organism depends on the quantity of available glucose."

This is a very suggestive, not to say startling, view-point, and is so important, if true, that we feel especial care should be given in considering alternative explanations which may not be of so revolutionary a nature. In one case 100 gm. of galactose were introduced into the stomach in solution in 300 cc. of water, while in the second experiment 100 gm. of galactose plus 100 gm. of glucose were introduced into the stomach in 600 cc. of water. Assuming some limit in the rate of sugar absorption for the digestive tract, it will be obvious that in the second experiment galactose could not have entered the blood stream nearly as rapidly as in the first instance. It might seem that the difference in the sugar elimination was too great to be covered by the possible difference in rate of absorption of the galactose, but there is evidence to show that this factor may adequately explain the results. The galactose lost is not a fixed proportion of the sugar given, in the sense that the organism can utilize just so much galactose and no more. The paradoxical law of sugar utilization (Allen) holds for this sugar like the others. Thus we note that when subject H. B-d. received 30 gm. of galactose in one dose, the loss of sugar through the kidney was less than one-tenth of that with 100 gm. of galactose. We are, therefore, inclined to feel that "dilution" of the galactose with the glucose may be regarded as an adequate explanation of the facts unless experimental evidence is offered which disproves this view.

In conclusion we wish to point out that our previous work clearly showed that under ordinary conditions, not more than about 50 per cent of the total sugar of normal urine can be glucose and that usually the *glucose cannot exceed 25 per cent of the total sugar*. MacLean (16) had previously reached a quite similar conclusion. He found that about one-half of the sugar of normal urine was removed by yeast. The fact that Folin and Berglund completely ignored the question of the action of yeast on the urine sugars does not alter our finding that fermentable sugar disappears from the urine during fasting, and appears after food intake. Pentoses are not destroyed by yeast. Traces of lactose or galactose may be, but our fermentable sugar was not dependent upon ingestion of milk. The presence or absence of a "motley" variety of sugars in the urine does not alter the fact of the appearance of increased sugar in the urine after the ingestion of food

free from carbohydrate (protein-fat, gelatin, and even olive oil, Folin and Berglund). Our findings (3) that pure glucose given on an empty stomach behaved quite differently from the identical quantity of the same glucose when given with a weighed meal, together with our observations on the effect of every meal upon the sugar output, led us to conclude that there is a real question of assimilation limit for sugar involved during the process of digestion and absorption. The work of Folin and Berglund would seem to corroborate and extend this view rather than to disprove it.

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ON A POSSIBLE ASYMMETRY OF ALIPHATIC DIAZO COMPOUNDS. IV.

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In previous publications under the same title, [1,2] experimental facts were reported which indicated the possibility of the existence of optically active aliphatic diazo esters. The various samples of diethyl diazosuccinate varied in their optical rotation between + 1 and 1.5°. The active diazo ester was converted into active diethyl malate, into active diethyl chlorosuccinate, as well as into diethyl bromosuccinate. However, we did not consider the evidence furnished through these substances conclusive for the following reasons. The diazo ester and all the hitherto described derivatives are liquids. They could be purified only by fractional distillation, and we seemed to have exhausted the possibility of further purifying them by this method. All the substances obtained in this manner possessed only a low degree of optical activity. Our efforts were therefore directed toward preparing either a crystalline diazo ester or converting the liquid diazo ester into a crystalline derivative of higher optical activity. After the publication of several of our papers on the active diethyl diazosuccinate and its derivatives, Chiles and Noyes[3] reported results similar to ours using as starting material instead of aspartic acid, its next higher homologue, glutamic acid. They also repeated our experiments on aspartic acid. In one point Chiles and Noyes seem to have made further progress than we, inasmuch as they claim to have reduced diethyl diazoglutarate to the corresponding amino-acid, possessing optical activity. Unfortunately, we could not corroborate the claim of Chiles and Noyes. As reducing agent we, as did Chiles and Noyes, employed either

[1] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1920–21, xlv, 593.
[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1922, lii, 485.
[3] Chiles, H. M., and Noyes, W. A., *J. Am. Chem. Soc.*, 1922, xliv, 1798.

aluminum amalgam or zinc dust and acetic acid. The reduction with aluminum amalgam was carried out in ethereal solution, or in ethereal solution which was maintained slightly acid by the addition of acetic acid from time to time. The resulting aspartic and glutamic acids were always inactive. There is another statement in the paper of Chiles and Noyes which we fail to understand. Starting from the natural *l*-asparagine, they obtain a levo-rotatory diazo ester which they converted into a levo-rotatory malic ester. We worked with the same substance for several years and consistently obtained dextro-rotatory diethyl diazosuccinate. This was converted invariably into dextro-rotatory malic ester. We wish to add that all our readings were taken in tubulated tubes.

Thus the solution of the question did not seem to have been advanced further through the work of Chiles and Noyes. We therefore continued our efforts to prepare an optically active crystalline derivative of diethyl diazosuccinate.

The conversion of the diethyl bromosuccinate into the free acid seemed to offer the greatest promise in this direction. Preference was given to the bromo derivative over the corresponding chloro derivative because of its lesser solubility. The conversion of the ester into the acid can be accomplished either by halogen halides, or other mineral acids, or by dicarboxylic organic acids. Sulfuric and oxalic acids proved unsatisfactory for this purpose as the hydrolysis by their aid proceeded very slowly and resulted in the conversion of the bromosuccinic ester into fumaric acid. The most satisfactory catalyst appeared to be aqueous hydrogen chloride. The hydrolysis was accomplished in 45 minutes, using a 10 per cent solution of hydrochloric acid. However, the hydrolysis was complicated by the formation of chlorosuccinic acid which could only be removed with great difficulty. The formation of the chlorosuccinic acid is easily explained by the intermediary formation of fumaric acid. It was, however, necessary to take into consideration also the possibility of converting optically active malic acid into optically active chlorosuccinic acid by boiling with hydrochloric acid. Experience of the past speaks against this possibility. Kekulé[4] observed that the conversion of malic acid into chlorosuccinic acid, by boiling with hydrogen chloride, proceeded very slowly, and also that the resulting substance was optically inactive. Nevertheless,

[4] Kekulé, *Ann. Chem.*, 1864, cxxx, 25.

we repeated the experiment by boiling optically active ethyl malate with hydrochloric acid, however, under the conditions which were employed for the hydrolysis of bromosuccinic acid. The result was entirely negative, as no chlorosuccinate was obtained. Thus it was evident that the optical activity of the halogen acids obtained on hydrolysis of bromosuccinic acid could not be attributed to the contaminating chloro derivative. However, the correctness of this statement was further tested in a very rigorous manner. Fortunately, the bromosuccinic acid possesses a much higher optical activity than the chlorosuccinic acid. The original product of hydrolysis consisted of bromosuccinic, chlorosuccinic, and fumaric acids, and had an optical rotation $[\alpha]_D^{20} = +0.2°$. 800 gm. of this material were prepared. The material was purified by fractional recrystallization from a mixture of ether and petroleum ether until a substance was obtained which had an optical rotation $[\alpha]_D^{20} = +60.45°$ and melted at 176°C. Bromosuccinic acid has a rotation $[\alpha]_D^{20} = +67.92$ and a melting point of 172°C. Our substance contained 35.96 per cent bromine and 2.35 per cent chlorine. Inasmuch as chlorosuccinic acid contains 23 per cent of chlorine and bromosuccinic acid 40 per cent of bromine, it is evident our substance was a mixture consisting of 10 per cent chlorosuccinic acid and 90 per cent bromosuccinic acid. If the optical rotation of the substance were due to the chlorosuccinic acid, the total rotation would have been $[\alpha]_D^{20} = +2.3°$; on the other hand, assuming that the rotation was due to the bromosuccinic acid, the mixture should have the rotation $[\alpha]_D^{20} = +61.3°$ (in ether) and in aqueous solution $[\alpha]_D^{20} = +39.87°$. These values agree very well with the observed rotation of the substance. Thus there seems to be no doubt that treatment of diethyl diazosuccinate in ether solution in the cold with gaseous hydrogen bromide gives rise to active bromosuccinic ester, and, therefore, one is justified in assuming that the optical activity of our diethyl diazosuccinic ester was due not to a contamination, but to the diazo ester itself. The explanation of the mechanism of the asymmetry we leave for a later date.

EXPERIMENTAL.

Preparation of Bromosuccinic Acid.—The diethyl bromosuccinate used in this experiment was prepared from undistilled

diethyl diazosuccinate as described in a previous paper.[1] In this case, however, the bromo ester was distilled only once and without further purification was subjected to hydrolysis. In this state of purity the bromo ester showed an optical rotation of $[\alpha]_D^{20} = +0.78°$.

90 gm. of the ester were hydrolyzed by boiling with 300 cc. of 10 per cent hydrochloric acid under return condenser for 45 minutes. The ester was then completely dissolved. The solution was now concentrated to about one-third its original volume. On cooling, about 13 gm. of crystalline substance separated. On further concentration two more fractions were obtained, weighing, respectively, 30 and 7 gm. The first fraction was practically insoluble in ether and consisted principally of fumaric acid, as shown by its melting point.

Fractions II and III were mixed and the rotation was determined in ether solution; which was found to be

$$\left[\alpha\right]_D^{20} = \frac{+0.1° \times 100}{1 \times 50} = +0.2°$$

The combined fractions were recrystallized from 36 cc. of water. 14 gm. of the acid were obtained with an optical activity of

$$\left[\alpha\right]_D^{20} = \frac{+0.25° \times 100}{1 \times 50} = +0.5°$$

When dry, this fraction was treated with ether and filtered. The residue was washed several times with ether. The combined ether filtrates were treated with enough petroleum ether to produce an opalescence. On standing, 7 gm. of crystalline substance were obtained which showed a rotation of $[\alpha]_D^{20} = +1.0°$. In a similar manner about 125 gm. of the acid with a rotation of $[\alpha]_D^{20}$ = about $+1°$ were prepared. By repeatedly dissolving it in ether and precipitating with petroleum ether, a fraction was obtained which melted at 176°C. and had a rotation of

$$\left[\alpha\right]_D^{20} = \frac{+2.81° \times 100}{1 \times 4.648} = +60.45°$$

in ether solution. In aqueous solution

$$\left[\alpha\right]_D^{20} = \frac{+0.41° \times 100}{1 \times 11.64} = +35.2°$$

0.1000 gm. substance: 0.0940 gm. AgCl + AgBr.
After treating with Cl: 0.0740 gm. AgCl.
Found. Br 35.96, Cl 2.35.

Preparation of Diethyl-α-Diazoglutarate.—10 gm. of diethyl glutamate hydrochloride were dissolved in 20 cc. of water and cooled to $-10°C$. To this were added 60 cc. of ether and 5 gm. of sodium nitrite dissolved in 10 cc. of water. 25 per cent sulfuric acid was then added in small portions. The ether layer was poured off from time to time. The treatment was continued with fresh quantities of ether and sulfuric acid. This was repeated until the ether layer remained colorless. The ether extracts were combined, washed first with a little 10 per cent sulfuric acid, then with sodium bicarbonate solution, and finally with 5 per cent sodium carbonate. The ether extract was first allowed to stand over calcium chloride for about 5 minutes and then for several hours over anhydrous sodium sulfate. The ether was finally removed under diminished pressure. The residue weighed 6 gm. Nitrogen was determined by the method described in a previous paper.[6]

0.1362 gm. substance was dissolved in 10 cc. of 50 per cent isopropyl alcohol.

2 cc. of this solution: 1.42 cc. N_2, $t = 25°C.$, $P = 757.5$ mm.

$C_9H_{14}O_4N_2$.	Calculated.	N 13.08.
	Found.	" 11.54.

This corresponds to 80.6 per cent of the diazo ester. It had the following optical rotation in ether:

$$[\alpha]_D^{24} = \frac{+1.35° \times 100}{1 \times 50} = +2.70°$$

Reduction of Diethyl-α-Diazoglutarate.—20 gm. of diethyl-α-diazoglutarate were dissolved in ether and reduced with aluminum amalgam. The amalgam was added from time to time in small amounts, the exhausted amalgam was removed before the addition of fresh amalgam. When all was reduced, the filtrate was treated with an ether solution of hydrochloric acid and the ether removed under reduced pressure. The residue was taken up with 100 cc. of 10 per cent hydrochloric acid, extracted with ether to remove the hydroxy ester, and heated under a reflux for 2 hours. The solution was now concentrated under diminished pressure to a small volume, saturated with hydrochloric acid gas, and allowed to stand for several hours at 0°C. The hydrochloride of glutamic acid

[6] Levene and Mikeska,[2] p. 488.

separated, and was recrystallized by dissolving in water and saturating with hydrochloric acid. The substance showed no optical rotation in water (c = 11.76; l = 1). The original mother liquor was combined with the mother liquor obtained from the recrystallization of the acid and concentrated to a volume of 10 cc. The solution in a 1 dm. tube showed no optical rotation.

Reduction of Diethyl Diazosuccinate.—20 gm. of diethyl diazosuccinate $[\alpha]_D^{20} = +1.01°$ were dissolved in ether and reduced with aluminum amalgam. The latter was added in portions of 2 gm. at a time, being freshly prepared for each addition. The exhausted amalgam was always filtered off before a fresh quantity was added. This procedure was repeated until the ether layer was perfectly colorless. During the last stages of the reaction a few drops of water were added from time to time. The reaction mixture was allowed to stand over night; the next day the amalgam was filtered off, the filtrate treated with a small quantity of hydrochloric acid dissolved in ether, and the ether removed under diminished pressure. The residue was treated with 10 per cent hydrochloric acid, extracted with ether to remove the hydroxy ester, and then heated for 2 hours under a reflux. On cooling the solution was again extracted with ether and subsequently concentrated under reduced pressure. The hydrochloric acid was removed by means of silver carbonate, and the silver in turn by hydrogen sulfide. The silver sulfide was filtered off and the filtrate concentrated under reduced pressure. On cooling, the aspartic acid crystallized. It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 7.25 cc. 0.1 N HCl.

$C_4H_7O_4N$.	Calculated.	N 10.52.
	Found.	" 10.15.

0.4306 gm. of the acid dissolved in 10 cc. of 1.22 N HCl showed no optical rotation in a 1 dm. tube.

In another experiment the reduction was carried out in an acid solution. That is, before the amalgam was added to the ether solution of the diethyl succinate, a little glacial acetic acid was added. From time to time enough acetic acid was added to keep the solution definitely acid toward litmus. The isolation and purification of the aspartic acid were carried out as described above. 11 gm. of aspartic acid were thus accumulated and the rotation was determined on a concentrated solution of the hydrochloride. No optical activity was observed.

CALCULATION OF ISOELECTRIC POINTS.

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The formula for calculation of the isoelectric point of a simple mono-basic, mono-acidic amphoteric substance is as follows:

$$I = \sqrt{\frac{K_a}{K_b} K_w} = \sqrt{K_a k_b} \quad \text{or } pI = \frac{pK_a + pk_b}{2} \tag{1}$$

Where I = the isoelectric point (hydrogen ion concentration at that point).

K_a = the equilibrium constant of the acid.

K_b = the equilibrium constant of the base.

K_w = the equilibrium constant of the water at the temperature under consideration.

$$k_b = \frac{K_w}{K_b}$$

It will be shown in this paper that this formula has an even wider application since in more complex ampholytes (as proteins) the isoelectric point may be approximately calculated from the equilibrium constants of the *strongest acid group* and the *strongest basic group* by the above equation.

A more exact equation is:[1]

$$I = \sqrt{\frac{K_{a1} + K_{a2} + K_{a3} \ldots . + K_{am}}{K_{b1} + K_{b2} + K_{b3} \ldots . + K_{bn}} K_w} = \sqrt{\frac{\Sigma K_a}{\Sigma K_b} K_w} \tag{14}$$

From this equation it is obvious that when the numerical value of the weaker K_as and K_bs is negligible in comparison with the strongest K_a and K_b, the equation resolves itself into equation (1). Thus if the second pK_a is *one pH unit* away from the first, it

[1] Even this latter equation is approximate, but it is accurate enough for practical use since the error is very small. The correct expression as applied to a di-basic, di-acidic ampholyte is given later in the paper.

may be neglected with small error. The weaker basic K_bs may be similarly disregarded.

The reason for assuming that the weaker acid and basic groups can be neglected may be demonstrated in the case of aspartic acid. Its dissociation is represented in Fig. 1. As determined by the authors, aspartic acid at 30°C. has the following constants.[2]

pK_{a1} =	3.63	K_{a1} =	$2.35 \cdot 10^{-4}$
pK_{a2} =	9.47	K_{a2} =	$3.39 \cdot 10^{-10}$
pk_b =	1.90	k_b =	$1.26 \cdot 10^{-2}$
pK_b (= $pK_w - pk_b$) =	11.82	K_b =	$1.50 \cdot 10^{-12}$
pK_w at 30°C. =	13.725	K_w =	$1.89 \cdot 10^{-14}$

These values were calculated from the titration data given in Table I, which were obtained for aspartic acid (molecular weight = 133) at 30°C., in 0.1 molar solution. The titration was at constant volume. A water-jacketed electrode was used which will be described in another publication. Near the isoelectric point, the values were obtained from solutions which were supersaturated.

If we consider only the stronger acid group, the calculation of the isoelectric point by equation (1) gives pI = 2.76. If this is the isoelectric point, the calculated hydrogen ion concentration for the dissolved substance in 0.1 N solution is pH 2.80.

In Fig. 1 the point I (indicated by the arrow) is the calculated isoelectric point where only the stronger acid group is considered. Calculation of the isoelectric point from equation (2) gives:

$$I = \sqrt{\frac{0.000235 + 0.000000000339}{1.50 \times 10^{-12}} \times 1.89 \times 10^{-14}}$$

$$pI = 2.76$$

[2] It will be shown in a later publication that the solubility of the *undissociated molecule* of aspartic acid has a constant value of 0.034 mol per liter at 25°C.

The *total* solubility can be calculated from the ratio:

$$S = \frac{0.034}{(1 - \alpha_a)\ (1 - \alpha_b)} = \text{total solubility.}$$

Where α_a and α_b equal the degree of dissociation of the first acid group and the basic group, respectively.

The value $(1 - \alpha_a)\ (1 - \alpha_b)$ represents the fraction of molecules not ionised.

TABLE I.

$\frac{B}{C} = \frac{-A}{C}$ Molar equivalents of base.	$B' = -A' = \frac{B+H}{C}$ Corrected for acid required to bring water to the same pH.	pH	pk_b	pK_{a1}	pK_{a2}
−1.0	−0.69	1.51	1.87		
−0.9	−0.64	1.61	1.88		
−0.8	−0.60	1.70	1.90		
−0.7	−0.53	1.82	1.90		
−0.6	−0.49	1.95	1.96		
−0.5	−0.42	2.09	(2.00)		
−0.4	−0.34	2.18	1.95		
−0.3	−0.25	2.31	1.92		
−0.2	−0.16	2.43	1.87		
−0.1	−0.07	2.60	(2.01)		
0	0.02	2.72	1.87		
+0.3		3.31		3.60	
+0.4		3.50		3.63	
+0.5		3.67		3.64	
+0.6		3.84		3.65	
+0.7		4.02		3.64	
+0.8		4.27		3.66	
+0.9		4.57		3.62	
+0.95		4.87		3.59	
+1.0		6.93			
+1.05		8.16			9.44
+1.10		8.48			9.43
+1.2		8.86			9.46
+1.3		9.11			9.48
+1.4		(9.47)			(9.64)
+1.5		9.580			(9.58)
+1.6		9.65			9.48
+1.7		9.85			9.48
+1.8		10.08			9.48
+1.9		10.36			(9.41)
+2.0		10.75			
Average of best values			1.90	3.63	9.47

pk_b is calculated from the equation $pk_b = pH + \log \frac{HA' + K_{a1}(1 + A')}{H(1 - A') - K_{a1} A'}$

pK_{a1} " " " " " $pK_{a1} = pH + \log \frac{k_b (1 - B') - HB'}{k_b B' + H(1 + B')}$

pK_{a2} " " " " " $pK_{a2} = pH + \log \frac{2 - B'}{B' - 1}$

The derivation of these formulas will be given in a future publication.

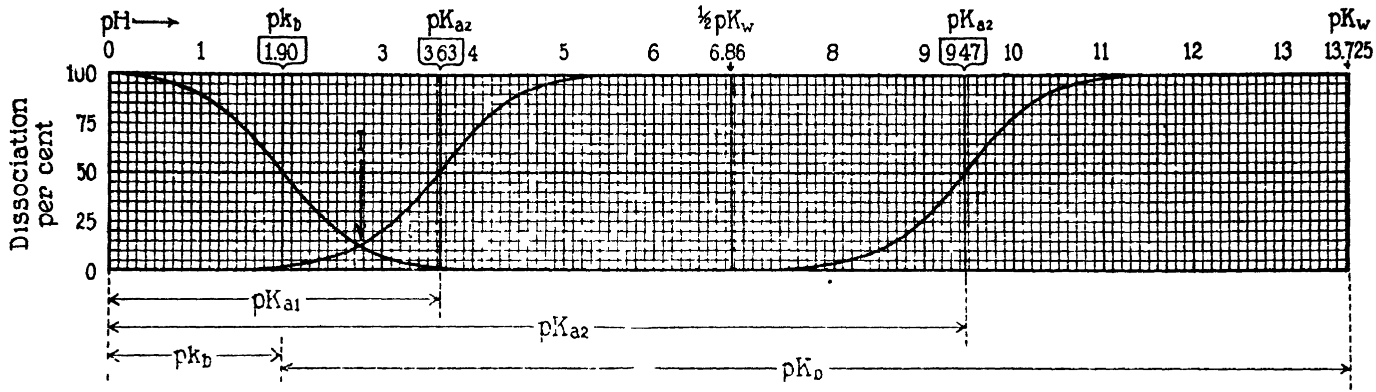

Fig. 1. Dissociation diagram of aspartic acid at 30°C.

This is the same value obtained by using equation (1) since the term K_{a2} is negligible in comparison with K_{a1}.

At the isoelectric point the first acid group is 15 per cent ionized, while the ionization of the second acid group is only 0.00002 per cent when calculated by the formula:

$$\text{pH} = \text{pK} + \log \frac{\alpha}{1-\alpha}$$

In other words, the first acid group of aspartic acid is nearly *one million times* as much ionized as the second. Hence the second may be neglected in calculating the isoelectric point.

The following values illustrate the relative accuracies of equations (1), (14), and (15).

Equation (1). $I = \sqrt{K_{a1}k_{b1}} = \sqrt{\frac{K_{a1}}{K_{b1}}K_w}$ or $pI = \frac{pK_{a1} + pk_{b1}}{2}$,

where only the strongest acid and basic groups are considered.

Equation (14). $I = \sqrt{\frac{K_{a1} + K_{a2}}{k_{b1} + k_{b2}} k_{b1} k_{b2}} = \sqrt{\frac{K_{a1} + K_{a2}}{K_{b1} + K_{b2}} K_w}$

Equation (15) (exact formula).

$$\sqrt{\frac{K_{a1} + K_{a2}}{k_{b1} + k_{b2}} k_{b1} k_{b2} + K_{a1}K_{a2}\left(1 + \frac{2k_{b1}k_{b2}}{H(k_{b1} + k_{b2})}\right) - \frac{H_2}{k_{b1} + k_{b2}}(K_{a1} + K_{a2} + 2H)}$$

as applied to two hypothetical di-basic, di-acidic ampholytes having the pK and pk values indicated.

TABLE II

Case No	pK_{a1}	pK_{a2}	pk_{b1}	pk_{b2}	pI = isoelectric point.			
					Equation (1)	Equation (14)	Equation (15)	True value.
1	3	5	2	1.00	2.500	2 520	2.531	2.531
2	4	10	2	1 43	3.000	3 052	3.060	3.060

Conception of Isoelectric Point.

In this paper the *isoelectric point* of an amphoteric substance will be interpreted to signify *that hydrogen ion concentration at which it is ionized equally as an acid and as a base.*

The isoelectric point may also be considered as the point of *minimum dissociation.* It will be seen later that both definitions lead to the same formula.

It is at this point that the conductivity will be a minimum, if the mobilities of the ampholyte are the same in the anion and cation forms.

Other properties such as optimum precipitation and agglutination, minimum viscosity, swelling, and solubility are associated with this degree of acidity or alkalinity which we call the isoelectric point.

It must be kept in mind that this point is *not* necessarily the same as the hydrogen ion concentration of a pure solution of the ampholyte.

At the *isoelectric point* the condition is represented by the equation

$$\alpha_{b1}C + \alpha_{b2}C + \alpha_{b3}C \ . \ . \ . + \alpha_{bn}C = \alpha_{a1}C + \alpha_{a2}C + \alpha_{a3}C \ . \ . \ . + \alpha_{am}C$$

or

$$\Sigma\alpha_b = \Sigma\alpha_a$$

while the hydrogen ion concentration of a solution of ampholyte is represented by

$$[H^+] + \Sigma\alpha_b C = [OH^-] + \Sigma\alpha_a C$$

where

α_b represents the degree to which a basic group is ionized.
α_a represents the degree to which an acid group is ionized.
C represents the concentration of ampholyte.
$[H^+]$ and $[OH^-]$ have the usual significance.

It will be seen that the *isoelectric point* as defined is *constant* and *independent of the concentration,* while the *hydrogen ion concentration* of the pure ampholyte solution is obviously a *variable* since it is a function of the concentration.

Except in the case where the isoelectric point happens to be at the "neutral point" of water, it is *never* identical with the hydrogen ion concentration of the dissolved substance. However, the difference is quite insignificant in concentrated solutions and is of notable magnitude in only quite dilute solutions.

Mode of Ionization of Ampholytes.

The following derivations of formulas for the calculation of isoelectric points are based on the assumption that the ionization

of each group (acid or basic) takes place *independently* of the *degree* of ionization of other groups in the molecule. It is understood that the numerical value of each K may be influenced by the relative position of other acidic groups. Mathematically, we may treat an ampholyte as if it were a mixture of a number of monovalent acids and bases having the same respective dissociation constants as the various groups of the ampholyte.

This conception will be more fully discussed in another publication. It is in accord with experimental data as far as the mass law is applicable.

Derivation of Formulas for the Calculation of the Isoelectric Point of Poly-Acidic, Poly-Basic Ampholytes.

If we define the isoelectric point of a substance as *that hydrogen ion concentration at which it is ionized equally as an acid and as a base*, we may represent this relation as follows:

$$\alpha_{b1}C + \alpha_{b2}C + \alpha_{b3}C \ldots + \alpha_{bn}C = \alpha_{a1}C + \alpha_{a2}C + \alpha_{a3}C \ldots + \alpha_{am}C \quad (2)$$

Where C represents the concentration of the ampholyte and the various values of α_b and α_a represent the extent to which the various basic an l acid groups are ionzed.

This may be written

$$\Sigma_n \alpha_b C = \Sigma_m \alpha_a C \quad (3)$$

or

$$\Sigma\alpha_b = \Sigma\alpha_a \quad (4)$$

that is, *the sum of the ionized fractions of basic groups equals the sum of the ionized fractions of the acid groups of an ampholyte* at its isoelectric point. It will be noted that the concentration factor (C) cancels out, thus making the isoelectric point *independent of the concentration.*

That this point is *not* identical with the hydrogen ion concentration of a *solution* of the *pure ampholyte* will be seen from the fact that this latter condition is represented by

$$[H^+] + \Sigma\alpha_b C = [OH^-] + \Sigma\alpha_a C \quad (5)$$

(for the sum of the positive ions in a solution must equal the sum of the negative ions).

Except in the special case where the isoelectric point is at the "neutral" point of water $[H^+]$ does not equal $[OH^-]$ and the pH of a pure solution of ampholyte lies between its isoelectric point and the neutral point of water. The exact pH is a function of concentration and in concentrated solutions its deviation from the isoelectric point is within experimental error.

1. Isoelectric Point of a Simple Ampholyte.

For the calculation of the pH at which

$$\Sigma\alpha_b = \Sigma\alpha_a \tag{4}$$

it has been customary to use the equation

$$I = \sqrt{\frac{K_a}{K_b} K_w} \tag{1}$$

where $n = m = 1$; that is, where there is but one acid group and one basic group.

This equation will be later deduced from a more general equation applying to cases in which there are more acid and more basic groups.

2. Isoelectric Point of a Poly-Basic, Poly-Acid Ampholyte.

The law of mass action for any mono-basic acid may be expressed as follows:

$$K_a = [H^+] \frac{\alpha_a}{1 - \alpha_a} \tag{6}$$

where K_a = the equilibrium constant,
$[H^+]$ = the hydrogen ion concentration,
α_a = the fraction of the acid in the ionized state.

Hence:

$$\alpha_a = \frac{K_a}{H + K_a} \tag{7}$$

Similarly, for a base:

$$K_b = [OH^-] \frac{\alpha_b}{1 - \alpha_b} \tag{8}$$

and

$$\alpha_b = \frac{K_b}{[OH^-] + K_b} \tag{9}$$

It will be recalled that the pK of an acid is the hydrogen ion concentration (expressed in terms of pH) at which the acid is half ionized.

For, if•

$$pK_a = pH = -\log K_a = -\log H$$

then

$$K_a = [H^+]$$

and

$$\frac{K_a}{[H^+]} = \frac{\alpha_a}{1 - \alpha_a} = 1$$

or

$$\alpha_a = 1 - \alpha_a \text{ and } \alpha_a = 0.5$$

So that at this point the numerical values of the dissociation constant and the hydrogen ion concentration are equal. Similarly, the pK of a base is equal to the p[OH⁻] or the negative logarithm of the *hydroxyl ion concentration* at which the base is half dissociated.

However, since we are accustomed to indicate acidity and alkalinity in terms of pH, pk_b may be used to indicate the pH at which the base is half dissociated.

The relation is

$$pk_b = pK_w - pK_b \tag{10}$$

or

$$k_b = \frac{K_w}{K_b} \text{ and } K_b = \frac{K_w}{k_b}$$

Substituting this in equation (9) and placing $[OH^-] = \frac{K_w}{[H^+]}$ we get

$$\alpha_b = \frac{[H^+]}{[H^+] + k_b} \tag{11}$$

Let us now take the case of an ampholyte with two acid and two basic groups whose ions are represented by α_{a1}, α_{a2}, α_{b1}, and α_{b2}. Their dissociation constants are: K_{a1}, K_{a2}, K_{b1}, and K_{b2} or K_{a1}, K_{a2}, $\frac{K_w}{k_{b1}}$, and $\frac{K_w}{k_{b2}}$. From equation (4) $\alpha_{b1} + \alpha_{b2} = \alpha_{a1} + \alpha_{a2}$; then from equations (7) and (11)

$$\frac{[H^+]}{[H^+] + k_{b1}} + \frac{[H^+]}{[H^+] + k_{b2}} = \frac{K_{a1}}{[H^+] + K_{a1}} + \frac{K_{a2}}{[H^+] + K_{a2}} \tag{12}$$

Solving this equation for [H+] involves a fourth power equation, so let us solve it in an approximate form and later derive the exact expression.

By way of approximation this may be written

$$\frac{[H^+]}{k_{b1}} + \frac{[H^+]}{k_{b2}} = \frac{K_{a1}}{[H^+]} + \frac{K_{a2}}{[H^+]} \tag{13}$$

Solving

$$I = [H^+] = \sqrt{\frac{K_{a1} + K_{a2}}{K_{b1} + K_{b2}} k_{b1} \cdot k_{b2}} \tag{14a}$$

or

$$I = [H^+] = \sqrt{\frac{K_{a1} + K_{a2}}{K_{b1} + K_{b2}} K_w} \tag{14b}$$

This is an equation which gives the approximate value of the isoelectric point of a di-basic, di-acidic ampholyte (see Table I). The exact solution of equation (12) gives (in terms of k_{b1} and k_{b2}):

$$\sqrt{\frac{K_{a1} + K_{a2}}{k_{b1} + k_{b2}} k_{b1} \cdot k_{b2} + K_{a1} \cdot K_{a2} \left(1 + \frac{2k_{b1} \cdot k_{b2}}{H(k_{b1} + k_{b2})}\right) - \frac{H^2}{k_{b1} + k_{b2}} (K_{a1} + K_{a2} + 2H)}$$

or (in terms of K_{b1} and K_{b2}):

$$\sqrt{\frac{K_{a1} + K_{a2}}{K_{b1} + K_{b2}} K_w + K_{a1} \cdot K_{a2} \left(1 + \frac{2K_w}{H(K_{b1} + K_{b2})}\right) - \frac{H^2 K_{b1} K_{b2}}{K_w (K_{b1} + K_{b2})} (K_{a1} + K_{a2} + 2H)}$$

It will be seen that the first terms in these equations are identical with the approximate equations (14a) and (14b). Hence it is to be expected that the sum of the remaining terms should be small or negligible. This is the case, and it will be seen from Table I that the error resulting from use of the simpler form is very small.

If it is desired to obtain a value for the isoelectric point which is of such accuracy that the error is entirely negligible, it is only necessary to obtain the approximate value of [H+] according to equation (14a) or (14b) and substitute this value for [H+] in equation (15a) or (15b). The value thus obtained is sufficiently accurate to be well within experimental error.

For an ampholyte having m acid groups and n basic groups, we saw that (equation (2)):

$$\alpha_{b1}C + \alpha_{b2}C \ldots . + \alpha_{bn}C = \alpha_{a1}C + \alpha_{a2}C \ldots . + \alpha_{am}C$$

or

$$\alpha_{b1} + \alpha_{b2} \ldots . + \alpha_{bn} = \alpha_{a1} + \alpha_{a2} \ldots . + \alpha_{am}C$$

Thus from equations (7) and (11)

$$\frac{[H^+]}{[H^+] + k_{b1}} + \frac{[H^+]}{[H^+] + k_{b2}} \ldots . + \frac{[H^+]}{[H^+] + k_{bn}} = \frac{K_{a1}}{[H^+] + K_{a1}} + \frac{K_{a2}}{[H^+] + K_{a2}} \ldots . + \frac{K_{am}}{[H^+] + K_{am}}$$

making the same approximation as in equation (13)

$$\frac{[H^+]}{k_{b1}} + \frac{[H^+]}{k_{b2}} \ldots . + \frac{[H^+]}{k_{bn}} = \frac{K_{a1}}{[H^+]} + \frac{K_{a2}}{[H^+]} \ldots . + \frac{K_{am}}{[H^+]}$$

Hence:

$$I = [H^+] = \sqrt{\frac{K_{a1} + K_{a2} \ldots . + K_{am}}{\frac{1}{k_{b1}} + \frac{1}{k_{b2}} \ldots . + \frac{1}{k_{bn}}}} = \sqrt{\frac{\Sigma K_a}{\Sigma \frac{1}{k_b}}} \qquad (14)$$

or

$$I = [H^+] = \sqrt{\frac{K_{a1} + K_{a2} \ldots . + K_{am}}{K_{b1} + K_{b2} \ldots . + K_{bn}} K_w} = \sqrt{\frac{\Sigma K_a}{\Sigma K_b} K_w}$$

This is the general approximate formula for the isoelectric point of an ampholyte with any number (m) of acid groups and any number (n) of basic groups (see Table I).

Equation (14) may be derived in a different manner on the assumption that at the isoelectric point the sum of all the ions ($\Sigma\alpha C$) is at a minimum. As in equation (13) we will take the approximate concentration of an acid ion, $\frac{K_aC}{[H^+]}$, and of a basic ion, $\frac{[H^+]K_bC}{K_w}$.

Then at the point of minimum concentration of ions:

$$\frac{d\Sigma\alpha C}{dH} = \frac{d}{dH}\left(\frac{K_{a1}C}{H} + \frac{K_{a2}C}{H} \ldots . + \frac{K_{am}C}{H} + \frac{K_{b1}CH}{K_w} + \frac{K_{b2}CH}{K_w} \ldots . + \frac{K_{bn}CH}{K_w}\right) = 0$$

Then

$$\frac{C}{H^2}(K_{a1}+K_{a2}\ldots.+K_{am})+\frac{C}{K_w}(K_{b1}+K_{b2}\ldots.+K_{bn})=0$$

or

$$\frac{\Sigma K_b}{K_w}-\frac{\Sigma K_a}{H^2}=0$$

Hence

$$I=H=\sqrt{\frac{K_{a1}+K_{a2}\ldots.+K_{am}}{K_{b1}+K_{b2}\ldots.+K_{bn}}K_w}=\sqrt{\frac{\Sigma K_a}{\Sigma K_b}K_w} \quad (14)$$

which is the same formula as the one obtained algebraically.

Substitution of this value in the second derivative

$$\frac{d^2\Sigma\alpha C}{dH^2}=\frac{2\Sigma K_a C}{H^3}$$

gives a positive value.

Hence equation (14) represents a minimum.

Position of Acid and Basic pK's.

It is not to be expected that all the acid pK's should occur above the isoelectric point and all the basic pk's ($= pK_w - pK_b$) should fall below this point. The above formulas apply to all cases, whether all the pK_a's are above and the pk_b's are below the isoelectric point, or whether there are some of each on both sides.

In any case where an acid pK does fall below the isoelectric point, there must be at least one basic pk above it.

It was found on titration of both aspartic acid (see Fig. 1) and glutamic acid that the solubility was lowest at the isoelectric point and increased on changing the pH in either direction. This may be ascribed to the insolubility of the undissociated molecule which is present in the largest proportion at the isoelectric point.[2]

Isoelectric Range.

While in aspartic acid, glutamic acid, and many proteins, the isoelectric point marks a sharp boundary where there is a definite change in behavior, this is not the case with all ampholytes. In the above cases the sharp change results from the proximity of

the acid and basic pK's to the isoelectric point. If, on the other hand, we consider glycine, alanine, leucine, etc., it will be seen that there is considerable range over which the ampholyte is dissociated. Thus between pH 4.5 and 8, the above three amino-acids are undissociated, exert no buffer effect, and behave in solution much as non-electrolytes. If we calculate the isoelectric point for such a substance, the value has no practical significance. There is in reality an isoelectric *range* from pH 4.5 to 8, in which there is no change in properties either physically or from the standpoint of ionization.

It is more proper, therefore, to speak of an isoelectric point only when pK_{a1} is less than 4 pH units above pk_{b1}.

SUMMARY.

1. The usual formula for single ampholytes:

$$I = \sqrt{\frac{K_a}{K_b} K_w} = \sqrt{K_a \cdot k_b}$$

may be used to calculate the approximate isoelectric point of poly-acidic, poly-basic ampholytes without much error by using the K_a and K_b of the strongest acid and strongest basic group.

2. A more exact expression is:

$$I = \sqrt{\frac{K_{a1} + K_{a2} \ldots . + K_{am}}{K_{b1} + K_{b2} \ldots . + K_{bn}} K_w} = \sqrt{\frac{K_{a1} + K_{a2} \ldots . + K_{am}}{\frac{1}{k_{b1}} + \frac{1}{k_{b2}} \ldots . + \frac{1}{k_{bn}}}}$$

$$I = \sqrt{\frac{\Sigma K_a}{\Sigma K_b} K_w} = \sqrt{\frac{\Sigma K_a}{\Sigma \frac{1}{k_b}}}$$

This will give a value with very small error.

3. The exact expression for the value of I is given.

4. The ionization of each acid or basic group is assumed to be independent of the degree to which all other groups are ionized at a given pH.

THE BIOS REQUIREMENT OF BAKERS' YEAST.*

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The question of the fundamental nutrition of yeast, and especially its vitamine requirements, has recently attracted considerable attention. The particular vitamine that has been hypothesized is generally called "bios." The following are the main questions that have been raised by the investigations so far: (1) Is bios identical with the water-soluble B vitamine of the higher animals? (2) Is bios necessary for the growth of yeast? (3) Where does yeast obtain its bios? (4) How does the bios function?

In the present paper data will be presented bearing on the first two questions; and the writers have kept in mind particularly the desirability of correlating their data with, and interpreting them in terms of, the results secured by other workers.

EXPERIMENTAL METHODS.

The medium used was essentially that of Clark (1), except that NH_4Cl was used instead of NH_4NO_3. The standard was:

	gm.
KH_2PO_4	5.0
$MgSO_4 \cdot 7H_2O$	2.5
$CaCl_2 \cdot 6H_2O$	0.35
Nitrogen (in combination)	0.5050
Sucrose, recrystallized from 80 per cent alcohol	100.0
Water up to	1,000 cc.

The first three salts were made up double strength for the stock solution. A stock solution of ammonium chloride, containing

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0.0347 gm. per cc., was also used. For each experiment 10 cc. of the double strength salts, 2 gm. of sucrose, a nitrogen compound equivalent to 0.0101 gm. N, and water to make a total of 20 cc., were added to a 100 cc. Erlenmeyer flask. When ammonium chloride was used with the nitrogen compound in question, nine-tenths of the 0.0101 gm. of N came from the former, and one-tenth from organic nitrogen compound. After adding beer-wort where indicated in the tables, the flasks were stoppered with cotton and sterilized at 12 pounds pressure for 10 to 15 minutes, cooled, seeded as indicated, and incubated at 30 ± 1°C. All cultures were grown in duplicate or triplicate.

The yeast cells were counted with a Zeiss hemocytometer. The large squares of the slide, each of which corresponds to $\frac{1}{250,000}$ cc., were used in the counts and usually 32 squares were counted from each drop. Whenever the counts were below 2 to 3 cells per square, usually 64 squares were counted from each drop, as this was found to give very close checks. The counts increase in accuracy inversely with their magnitude and are dependable within 10 per cent, except on very large counts where bunching makes exact counting impossible.

The yeast culture used was originally obtained from a Fleischmann cake and had been repeatedly pure cultured through many successive platings. This strain showed less bunching than other strains tried, one of which was originally obtained from Dr. F. W. Tanner. In every case a 48 hour agar slant culture was used to make up a convenient suspension in distilled water. For seeding, a pipette giving 40 drops per cubic centimeter was used; the calculated number of drops being added to give the initial count desired.

Non-Identity of Bios and Water-Soluble B.

In 1901 Wildiers (2) published a paper in which he called attention to the dispute between Pasteur and Liebig relative to the possibility of obtaining growth and fermentation by inoculating a synthetic medium with "une tête d'épingle" of yeast. Wildiers explained this disagreement by showing that *small* seedings of yeast will not grow on purified media without the addition of a substance which he provisionally called "bios" and which he

found to be present in yeast extract, beer-wort, and other biological materials. The yeast must have this substance added either in the form of surplus yeast cells or else in the form of extracts. Table I, taken from Wildiers' article, shows the influence of yeast extract when equal portions of purified media are inoculated with small seedings and treated with varying amounts of yeast extract.

Williams in 1919 (3) came to the conclusion: "A substance of unknown nature, which is a constituent of yeast, is necessary in addition to the ordinary nutrients for the nutrition of yeast cells." This substance he concluded to be identical with water-soluble vitamine B, and proposed to use the rate of yeast growth as a test for that vitamine. Miss Bachmann (4) arrived at similar conclusions, but proposed to use the amount of CO_2 liberated as an indicator of the vitamine effect.

TABLE I.

Wildiers' Data on the Effect of Yeast Extract upon the Fermentation with Small Seedings of Yeast.

Time.	Cc. of boiled yeast emulsion.				
	1	2	3	4	5
days	*cc.* CO_2	*cc.* CO_2	*cc.* CO_2	*cc.* CO_2	*cc.* CO_2
2	0	0	0.5	1.2	2.5
3	0	0	1.0	2.1	4.7
4	0	0	1.2	3.0	5.6

It was soon shown, however, that neither method was reliable (5, 6, 7), and the conclusion seems to be warranted that bios and water-soluble B are not the same, because of the following considerations.

(*a*) *Vitamine B is readily removable by 95 per cent alcohol, while bios is not.* In order to test the efficiency of different strengths of ethyl alcohol in purifying commercial sucrose for use in media, the writers used 95 per cent and 80 per cent alcohol, and the method of MacDonald and McCollum (8). The boiling alcohol was saturated with standard granulated sugar under a reflux, filtered hot, and allowed to crystallize. The crystals were filtered, and washed with the particular strength of alcohol involved. MacDonald and McCollum's sugar "was prepared from the best granulated cane sugar by repeated precipitation from a concen-

TABLE II

A Comparison of the Bios Contamination of Standard Granulated Sugar, and of the Same Crystallized from Various Strengths of Alcohol by Various Methods

Sucrose used	Daily record of growth in $\frac{\text{millions}}{4}$ per cc						
	0	1	2	3	4	5	14
Standard granulated	0.4		1.8		13	22	
" "	0.4		1.4		12	23	
" " crystallized from 95 per cent alcohol	0.4		1.3		12	22	
" " " " 80 " " " once	0.4		0.9		6	15	
" " " " 80 " " " twice	0.4		0.9		0.6	0.5	
" " " " 80 " " " thrice	0.4		0.8				
" "	0.4	1.2	2.5	7		27	32
" " crystallized once from 80 per cent alcohol	0.4	0.7	0.9	1.3		3	
" " " " " 80 " " " + 10 drops beer-wort	0.4	50	61	98		97	
Standard granulated crystallized once from 80 per cent alcohol + 20 drops beer-wort	0.4	90	125	117		115	
Standard granulated	0.5		12	32			
" " recrystallized from 80 per cent alcohol once	0.5		4	8			
" " " " 80 " " " twice	0.5		3	6			
" " " " 80 " " " thrice	0.5		3	5			
Method of MacDonald and McCollum*	0.5		14	32			
	0.5		12	31			
" " " " " †	0.5		11	24			
	0.5		9	21			

* First portion of crystals to come down

† Second " " " " " "

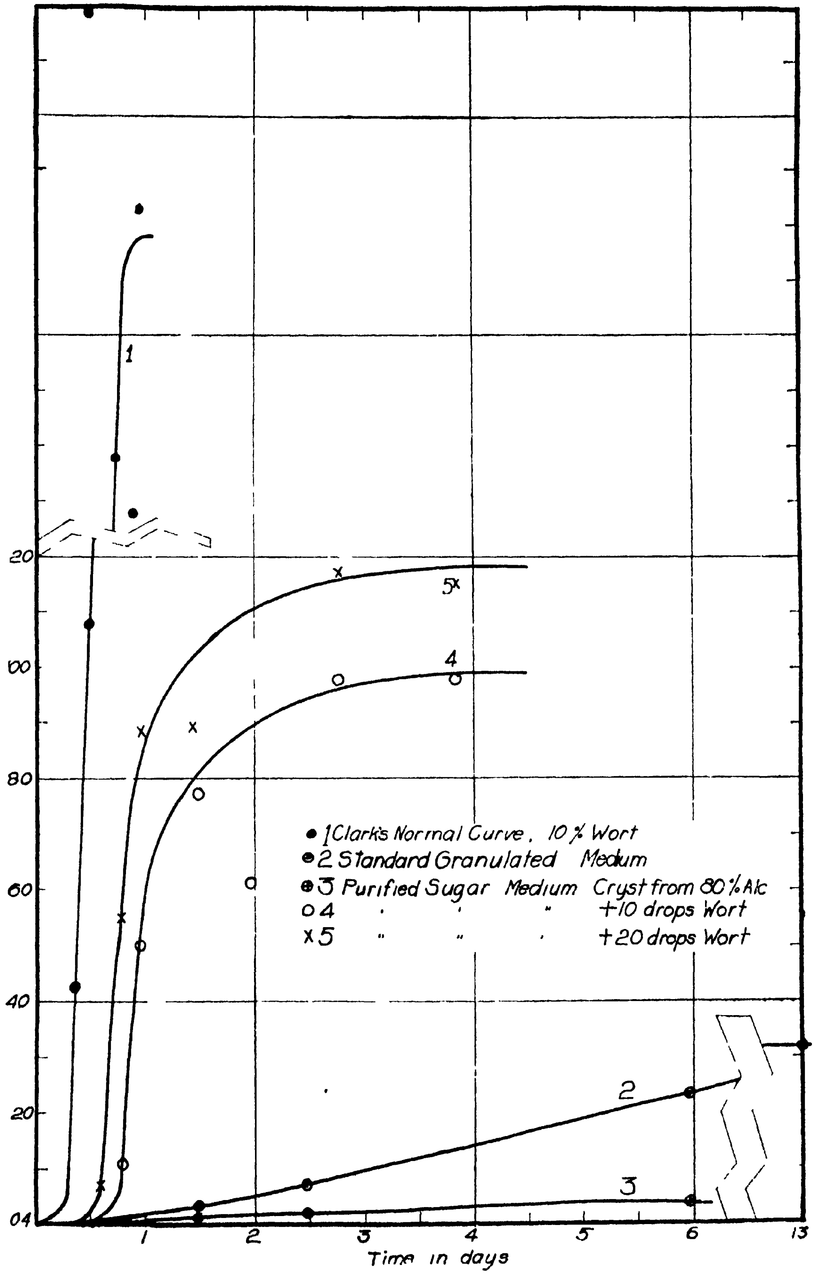

FIG. 1. The growth curves of yeast on wort and on synthetic media, with various sources of sugar and various amounts of bios.

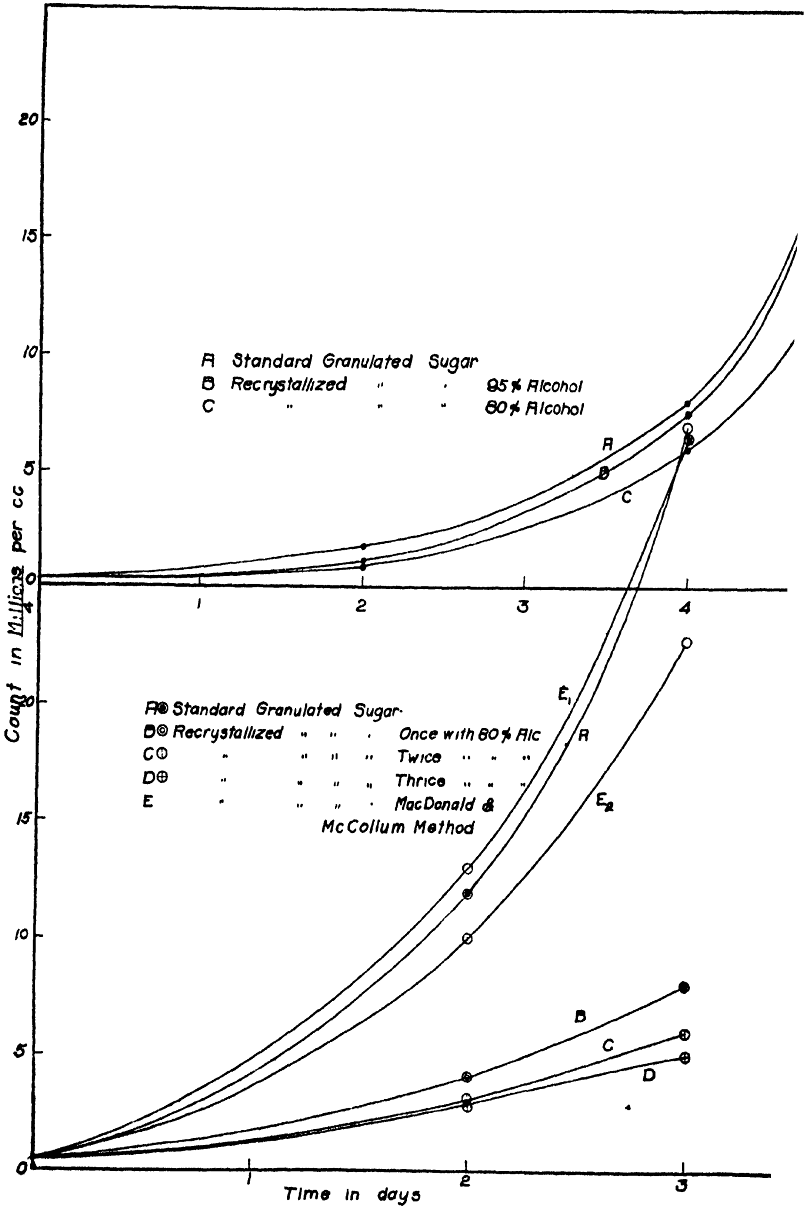

FIG. 2. The growth curves of yeast on synthetic media containing sucros crystallized from alcohol in various ways.

trated water solution by absolute alcohol." The growth of yeast was used as the criterion of freedom from bios. The results are presented in Table II and Figs. 1 and 2. It is evident that 95 per cent alcohol is a poor solvent for bios, but that 80 per cent is very good. The method of MacDonald and McCollum does not remove bios from commercial sugar, although it is an excellent method for the removal of vitamine B. This is the explanation of the controversy between Eddy, Heft, Stevenson, and Johnson (9), and Fulmer and Nelson (10). It also explains why Ayers and Mudge (11) caused stimulation of growth of streptococci with the residue left after the extraction of yeast with 95 per cent alcohol, as Funk and Dubin (12) had previously proved that streptococci are not stimulated by the same vitamine that is required by pigeons, but are stimulated by the same extracts that are required for yeast growth.

(*b*) *Bios is less readily adsorbed by fullers' earth than is water-soluble B.* Fullers' earth has been used as a method for the quantitative removal of the B vitamine by several investigators, but Fränkel and Scharf (13) proved that it is a poor adsorptive agent for the yeast stimulant; and Funk and Dubin (12) used this differentiation as a method of separating the two.

(*c*) It is well recognized that water-soluble B is not stable in dilute boiling alkalies, but several investigators (3, 7, 14) have found that *extracts thus treated still retain their potency for yeast growth.* This leads to the conclusion that there are two substances involved.

The Necessity of Bios for the Normal Growth of Bakers' Yeast.

Fulmer, Nelson, and Sherwood (7) and MacDonald and McCollum (8) contend that yeast not only does not require vitamine B, but that it also does not require bios for growth. The proof depends upon obtaining a medium completely free from unknown substances, especially those of a biological origin which might come under the term bios. The sugar used is the most likely source of such contamination. The first of the above investigators do not state how they purify their sugar, or that they purify it at all, and the latter purify theirs in the manner described above.

The writers undertook to prepare bios-free sugar by means of various concentrations of alcohol. Part of the results has been referred to in the section above; they will all be found in Table II and Figs. 1 and 2. In using the method of MacDonald and McCollum, when the absolute alcohol was poured into the sirup the first crystals to form were kept separate from the later and larger crop. The results show that the first crop carries considerably more yeast growth stimulant than the second, and that the combined fractions would carry practically as much as the original material. It is very doubtful whether several such fractionations would yield a sugar free from bios. Culture media containing standard granulated sugar give a count after 5 days of about 21; sugar prepared from 95 per cent alcohol gives about the same count, while that prepared from 80 per cent gives counts of from 3 to 15, depending upon the conditions of crystallization, and second and third recrystallizations from 80 per cent give still lower counts.

From these results the writers believe that there are no grounds for the opposition of the above workers to the bios hypothesis. There remains to be considered then, the evidence favoring the bios hypothesis.

In animal nutrition work the growth curve on the optimum diet is used as a standard by which to judge the performance of animals on experimental diets. The writers believe that the same principle should be adhered to in other lines of nutrition work. If the rapidity of yeast reproduction is at its maximum in beer-wort, the latter may be taken as a standard by which to measure all other media, or at least a goal to be striven for in building up a synthetic medium. The comparative slope of the curve is an index of the success of the medium.

Since bios belongs in the same category of nutritive substances as the animal vitamines, the proof of its existence must involve: (*a*) evidence that its absence from a medium otherwise complete prevents the normal growth of yeast; (*b*) its preparation in a form such that its addition to a known medium will make the latter essentially as good as beer-wort for yeast growth; and (*c*) evidence that it does not contribute directly to the mineral, energy, or nitrogen requirements of yeast.

The writers believe that the first condition has been fully met; but they admit that the evidence for the second and third is as yet circumstantial.

(a) Growth of Yeast without Bios.

Without recounting in detail all the recent cultural work with yeast bearing on this point, suffice it to say that practically all of it shows that yeast growth is infinitely slower on media made up of pure chemicals, and that the purer the latter are, especially from contamination with biological material, the poorer is the yeast growth. Fig. 1 shows a comparison of the rate of yeast growth in beer-wort and in various media of known composition. The writers' work described above, on the purification of sucrose from adhering molasses, indicates how difficult it may be to prepare materials pure with respect to yeast growth. Furthermore, in the work reported by Fulmer, Nelson, and Sherwood (7) and MacDonald and McCollum (8), in which the yeast was grown for many generations in "pure" media, and in which the original supply of bios was greatly attenuated, the growth was far from normal. And as the yeast grown on such media is abnormally low in vitamine B, as shown by Harden and Zilva (15), by Eijkman, van Hoogenhuijze, and Derks (16), by Darrah (17), and by Nelson and coworkers (18), it is probable that it is abnormal in content of bios also. The only contrary experience is that of Miss MacDonald (19), who believes that yeast synthesizes its own vitamine B. Furthermore, since Kennedy and Palmer (20) have shown that yeast is a very irregular source of vitamine B, depending upon its functional condition and on the medium in which it is grown, it is likely that its content of bios varies likewise; and indeed both Wildiers (2) and Amand (21) found that extracts of yeast grown on deficient media contained little or no bios.

(b) Identity of Bios.

Fractionation of bios from autolyzed yeast has been effected to a certain degree by Funk and Dubin (12), although they name the bios fraction vitamine D, and by Fränkel and Scharf (13). Its properties as far as known at present are as follows: it is soluble in water, soluble in 80 per cent alcohol, but sparingly

extracted from materials by 95 per cent; it is stable towards dilute boiling alkali (3, 7, 14); it is precipitated by phosphotungstic acid, but soluble in excess (2, 3, 13); it is adsorbed by fullers' earth, but much more weakly than is vitamine B (12); it is completely precipitated by mercuric chloride (13); and it readily dialyzes through a membrane (2, 22). These facts have aided in its separation from various sources in fairly concentrated preparations, and should enable investigators to succeed in adding it to synthetic media in such quantities as to make the latter equal to beer-wort in nutritive value for yeast.

Another method of arriving at the identity of bios is obviously to feed known compounds to yeast in the hope of happening on one that will simulate bios in its effect. This method has been used to a considerable extent, but so far without success, in identifying bios. Since this work is inseparably connected with that of investigations on the function of bios, the two phases will be considered together below.

(c) *Function of Bios in Yeast Growth.*

Evidence that bios does not contribute directly to the mineral, nitrogen, or energy requirements of yeast is somewhat difficult to establish. The energy phase can be ruled out because of the extremely small quantities of material required to show potency. As for minerals, the nutrition of yeast in this respect has been so thoroughly studied during the last 50 years that it appears unlikely that the properties of bios, as described above, could pertain to a mineral element. It is more plausible that bios may contribute a particular nitrogen group, which the yeast cell is unable to synthesize at all, or only to a limited extent. Fleming (6) has suggested that "the addition of organic nitrogen to the inorganic nitrogen of the culture medium is one factor in the stimulation of yeast growth." The great bulk of nutrition experiments on yeast, both when the bios hypothesis was kept in mind and also when it was not, goes to show that ammonia nitrogen is perfectly available to yeast as a source of nitrogen for growth; and that amino nitrogen is likewise utilizable, but that it is probably no better as a source of nitrogen than ammonia, nor a supplement to the latter.

It is probable, therefore, that for the growth of yeast cells ammonia nitrogen is quite satisfactory. Nevertheless, it might be possible that a small amount of some particular nitrogen group is necessary as an adjuvant to the gross nitrogen requirements. Even if this be so, it would not necessarily remove this substance from the class of vitamines, because the latter have not been proved to be other than substances incapable of synthesis by the animal, required in very small amounts, and probably containing nitrogen.

Table III is a list of compounds which have been used by various workers in yeast media under such conditions as would throw light on the bios proposition. By this is meant that all the substances in the media were known and substantially pure, and that the seeding of yeast was so small that growth without the compound in question was inappreciable.

The writers have used nineteen compounds of nitrogen in yeast cultures, with and without ammonium chloride, and with and without small amounts of beer-wort. Thus, the growth of the yeast under the various conditions would show whether a given compound can supply nutritive nitrogen, whether it is toxic, and whether additional bios improves its effect. The data are given in Tables IV and V. The experiments in Table IV were the first ones made, and the sequence of procedures was not quite the same as in the later ones.

In considering the data, the importance of the size of the seeding should be kept in mind. Clark (1) has shown conclusively that with optimum bios concentration the rate and final amount of yeast growth is independent of the original seeding. Others (2, 3, 23) have shown that with pure, synthetic media, very small seedings will not grow, whereas larger seedings will; and that the threshold seeding bears some relation to the bios content of the medium. In the writers' experiments, successive seedings of from 200 to 1,000 cells per cubic centimeter were used, allowing from 3 to 11 days for growth to become apparent. In Table V the letter x indicates seedings, and the figures indicate the average count of yeast cells in 1 large square of a Zeiss hemocytometer ($C \times 0.25$ = millions per cubic centimeter). The controls consist of the cultures with beer-wort but no other source of nitrogen, of those with ammonium chloride alone, and of those with beer-

TABLE III.

Nitrogen Compounds Investigated by Various Workers and Found Not To Be Bios.

Yeast growth method.		
Funk and Dubin (12).	**Swoboda (21).**	**Willaman and Olsen.**
Allantoin.	Arginine.	Ammonium chloride.
Hydantoin.	Asparagine.	Aspartic acid.
Leucine.	Cystine.	Alloxan.
Nicotinic acid.	Edestin (hydrolyzed).	Allantoin.
Pilocarpin.	Glucosamine.	Biuret.
Tryptophane.	Histidine.	Caffeine.
Mixture of 6 amino-acids.	Lysine.	Gelatin.
Mixture of 10 purine and pyrimidine bases.	Proline.	Guanine HCl.
	Tryptophane.	Guanidine HCl.
	Tyrosine.	Nucleic acid.
		p-Nitrophenol.
		Picramic acid.
		Piperidine.
		Potassium nitrate.
		Theobromine.
		Tyrosine.
		Thymine.
		Thiocarbamide.
		Uracil.
		Urea.
		Uric acid.

CO_2 method.		
Wildiers (2).	**Abderhalden (22).**	**Neuberg and Sandberg (27).**
Adenine.	*d-l*-Adrenalin.	Adenine.
Aniline.	Acetylcholine.	Allantoin.
Asparagine.	Allantoin.	Barbituric acid.
Creatine.	*d*-Arginine.	Barium mesoxalate.
Edestin.	Caffeine.	Guanine (natural and synthetic).
Guanidine.	Cholesterol.	Guanosine.
Nucleic acid (thymus).	Choline.	Hypoxanthine.
Nuclein bases.	Cysteine.	Heteroxanthine.
Ovalbumin.	*l*-Cystine.	8-Methylxanthine.
Tyrosine.	Diethylamine.	Monosodium urate.
Urea.	1-3-5-Diiodotyrosine.	Nucleic acid (thymus, yeast, spleen, sperm).
	1-3-4-Dioxyphenylalanine.	Parabanic acid.
	Histidine.	Potassium urate.
	Homovanillin.	Tetramethylxanthine.
	Tryptophane.	Theobromine.
	Tyrosine.	Theophylline.
	Vanillin.	Trichlorotetramethylxanthine.
	Xanthine.	Uric acid.

TABLE IV.

A Comparison of the Value of Different Nitrogen Compounds for Promotion and Growth in Yeast.

	Daily record of growth in $\frac{\text{millions}}{4}$ per cc																Appearance of cells.
	0	1	2	10	11	12	17	18	19	20	21	22	23	24	25	26	
Ammonium chloride	x*	–	–	x†	–	–	x‡	–	–	–	–	–	x§	6	20		Normal.
" "	x*	–	–	x†	–	–	x‡	–	–	–	–	–	x¶	–	–	–	
" " + 15 drops beer-wort	x*	–	–	x†	–	80											Normal.
Aspartic acid	x*	–	–	x†	–	–	x‡	–	3	8	15	27	29				"
" " $+NH_4Cl$	x*	–	–	x†	–	–	x‡	–	–	–	0.1	–	1.7	1.8	13		"
p-Nitrophenol	x*	–	–	x†	–	–	x‡	–	–	–	–	–	–				
" $+ NH_4Cl$	x*	–	–	x†	–	–	x‡	–	–	–	–	–	x§	–	–	–	
Potassium nitrate	x*	–	–	x†	–	–	x‡	–	–	–	–	–	x§	1.2	20		Abnormal.
" " $+ NH_4Cl$	x*	–	–	x†	–	–	x‡	–	–	–	–	–	x§	2.1	50		Normal.
Picramic acid	x*	–	–	x†	–	–	x‡	–	–	–	–	–	–				
" " $+ NH_4Cl$	x*	–	–	x†	–	–	x‡	–	–	–	–	–	x§	–	Growth.		Normal.
Tyrosine	x*	–	–	x†	–	–	x‡	–	–‖	–	–	–	–	25			"
" $+NH_4Cl$	x*	–	–	x†	–	–	x‡	–	–	–	–	–	–				
No nitrogen, but 6 drops beer-wort	x§	–	8	10	20												Abnormal.

* Seeded with approximately 200 cells per cc.
† " " " 400 " " "
‡ " " " 1,300 " " "
§ " " " 1,000 " " " + 6 drops beer-wort.
¶ " " " 1,000 " " "
‖ 10 drops beer-wort added.

TABLE V.

Comparison of Different Nitrogen Compounds for Promotion of Growth in Yeast.

	Drops of wort added	Daily record of growth in $\frac{\text{millions}}{4}$ per cc.																			Appearance of cells.
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
Ammonium chloride..........	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—	x	—	—	
	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	—			
	8	x	—	—	—	—	—	—	—	—	x	—	—	—	x	?	50				Normal.
Allantoin..................A	8	x	—	—	—	—	—	—	—	—	x	10	45	—	—	—					"
	6	x	—	—	—	—	—	—	—	—	x	2	40	—	—	—					"
" +NH_4Cl........B	8	x	—	—	—	—	—	—	—	—	x	0.2	80	—	—	—					"
	6	x	—	—	—	—	—	—	—	—	x	0.4	75	—	—	—					"
" C	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	2.6			"
" +NH_4Cl.......D	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	—			
Alloxan..................A	8	x	—	?	29	41	—	—	—	—	40										Normal.
	6	x	—	?	30	35	—	—	—	—	33										"
" +NH_4Cl........B	8	x	—	?	18	72	—	—	—	—	70										"
	6	x	—	?	18	63	—	—	—	—	63										"
" C	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	—			
" +NH_4Cl.........D	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	0.2			
Aspartic acid..............A	8	x	—	—	0.1	14.2															Normal.
	6	x	—	—	—	14.4															"
" " +NH_4Cl....B	8	x	—	—	2.5	32															"
	6	x	—	—	0.1	8															"
" " C	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	0.54	5.5		"
	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—	x	—	0.4	"

Biuret........................A	8	x	—	—	x	—	—	x	1	25	33	—	—								Abnormal.
	6	x	—	—	x	—	8.2	51	—												"
" +NH_4Cl.. ....B	8	x	—	—	x	—	—	x	—	—	—	—	x	—	—	65	65				Normal.
	6	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
" C	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
" +NH_4Cl............D	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
Gelatin.....................A	8	x	—	—	x	—	22	24													Normal.
	6	x	10	40	40																"
" +NH_4Cl.. ....B	8	x	0.9	110																	"
	6	x	—	—	x	—	7.6	67													"
" C	0	x	—	—	x	—	—	x	—	—	—	—	x	—	?	3.9	5.1	6.8			"
" +NH_4Cl..........D	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—	—			
Guanine HCl...............A	8	x	—	—	—	—	—	—	—	—	x	5.1	55	—	—	—	50	—			Abnormal.
	6	x	—	—	—	—	—	—	—	—	x	1.0	35	—	—	—	36	—			"
" +NH_4Cl........B	8	x	—	—	—	—	—	—	—	—	x	0.05	85	—	—	—	80	—			Normal.
	6	x	—	—	—	—	—	—	—	—	x	?	80	—	—	—	75	—			"
" C	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	—			
" +NH_4Cl........D	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	—			
Guanidine HCl... ....A	8	x	—	—	—	—	—	—	—	—	x	4	40	—	—	—	40	—			Abnormal.
	6	x	—	—	—	—	—	—	—	—	x	3	34	—	—	—	36	—			"
" +NH_4Cl........B	8	x	—	—	—	—	—	—	—	—	x	0.7	70	—	—	—	70	—			Normal.
	6	x	—	—	—	—	—	—	—	—	x	?	12	60	—	—	65	—			"
" C	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	1.4			Abnormal.
" +NH_4Cl.......D	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	—			

x Seeded to approximately 200 cells per cc., or a count of 0.001.

— No change observed.

TABLE V—*Concluded.*

	Drops of wort added	Daily record of growth in $\frac{\text{millions}}{4}$ per cc																			Appearance of cells.
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
Nucleic acid.... .. ... . A	8	x	45	66	—	—															Abnormal.
	6	x	45	72	—	—															"
" " +NH_4Cl .....B	8	x	3.7	100	—	—															Normal.
	6	x	1.2	75	—	—															"
" " C	0	x	4.3	5.3	—																Abnormal.
" " +NH_4Cl .. .D	0	x	—	—	x	—	—	x	—	—	—	—	x	10.8	28	—	29	—			Normal.
Piperidine.... ....A	8	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
	6	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
" +NH_4Cl . ...B	8	x	—	—	x	—	—	x	—	—	—	—	x	—	?	75	70				Normal.
	6	x	—	—	x	—	—	x	17	70	71										"
" C	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
" +NH_4Cl ......D	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
Theobromine ....A	8	x	—	—	x	—	—	x	—	40	37										Normal.
	6	x	—	—	x	—	—	x	—	23	25										"
" +NH_4Cl.....B	8	x	—	—	x	—	—	x	—	—	—	—	x	—	—	40	40				"
	6	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
" C	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
" +NH_4Cl....D	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
Thymine............... .A	8	x	3.2	80																	Normal.
	6	x	0.1	21																	"

Thymine +NH_4Cl.... B	8	x	—	—	x	—	—	x	?	46	45										Normal.
	6	x	—	—	x	—	—	x	—	—	—	—	x	?	34	53					“
“ C	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
“ +NH_4Cl.........D	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
Thiocarbamide.......... ..A	8	x	—	—	—	—	—	—	—	—	x	?	33	32	—	—	32	—			Abnormal.
	6	x	—	—	—	—	—	—	—	—	x	?	14	31	—	—	30	—			“
“ +NH_4Cl. B	8	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	75	—			Normal.
	6	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	—			
“ C	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	—			
“ +NH_4Cl ..D	0	x	—	—	—	—	—	—	—	—	x	—	—	—	x	—	—	—			
Uracil.................... A	8	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
	6	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
“ +NH_4Cl.... ... B	8	x	—	—	x	—	—	x	—	75	75										Normal.
	6	x	—	—	x	—	—	x	—	?	26										“
“ C	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
“ +NH_4Cl ....D	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
Urea.......................A	8	x	—	—	x	—	2.7	22	—	—	—	—									Normal.
	6	x	—	—	x	—	—	x	5	25	—	30									“
“ +NH_4Cl............B	8	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
	6	x	—	—	x	—	—	x	—	16	60	—	60	—	—	—	—				Normal.
“ C	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				
“ +NH_4Cl..... .....D	0	x	—	—	x	—	—	x	—	—	—	—	x	—	—	—	—				

wort alone. With the beer-wort alone as a source of nitrogen, there was invariably good multiplication of cells, but the cells were extremely small, and thickly bunched. With ammonium chloride alone, growth was seldom obtained, and then only after several thousand cells per cubic centimeter had been added in various seedings. When a little beer-wort was present, the ammonium chloride caused immediate growth, and the cells were large.

TABLE VI.

Summary of Conclusions from the Data in Tables IV and V.

	Produces normal cells without NH_4Cl, but with beer-wort. A.	Produces some stimulus, probably not due to bios contamination. A and B.	Probably contaminated with bios. C and D.
Ammonium chloride	+	−	−
Aspartic acid	+	+	−
Alloxan	+	+	−
Allantoin	+	+	−
Biuret	−	+	−
Gelatin	+	+	+
Guanine HCl	−	+	−
Guanidine HCl	−	+	−
Nucleic acid	−	+	+
p-Nitrophenol	−	−	−
Picramic acid			−
Piperidine	−		−
Potassium nitrate	−		−
Theobromine	+	+	−
Tyrosine	+		−
Thymine	+	+	−
Thiocarbamide	−	+	−
Uracil	−	−	−
Urea	+	+	−

The shortest way to summarize the data in the tables is to collect the conclusions under three headings, as in Table VI. The second column indicates whether the compound in question functions as an adequate source of nutritive nitrogen, with bios supplied. These conclusions are obtained from the cultures marked A in Table V. The third column is obtained by comparing the results in Cultures A and B in Table V. It will be noticed, in the A series, that growth usually is apparent a day or so earlier

than it is in the B series; and that growth in A reaches a maximum in a much shorter time than in B, but that this maximum is lower than the maximum in B. This difference is brought out graphically in Fig. 3. Since a comparison of C and D indicates any bios contamination, and since a majority of the compounds show no such contamination, the conclusion is that there is some stimulus to yeast growth other than a bios stimulus furnished by the compound. Other investigators (12, 22, 24) have noted the same phenomenon.

Attention should be drawn to the fact that a distinction is usually apparent between the cultures containing 8 drops, and those containing 6 drops, of beer-wort. This, together with the number of seedings required to induce growth, is an indication of the very low level of bios in these media.

Incomplete data were also obtained concerning caffeine and uric acid; they show that the former will support normal growth with bios added, but that the latter will not.

Taking into consideration all the available evidence, there is no question but that ammonium nitrogen is the best single source of nutritive nitrogen for yeast growth, so far observed, when adequate bios is supplied.

Whatever inferences may be drawn from the above data, the outstanding conclusion is that none of the compounds investigated can possibly be bios itself.

There is some evidence that bios may not function in yeast growth directly, but that it may assist the enzymes in their functions. Thus, several European authors have investigated the increase in CO_2 liberated by yeast zymase by addition of extracts from biological sources (22, 25, 26), by addition of various organic compounds such as might be present in yeast autolysates (22, 27), as well as such unrelated substances as charcoal and toluene (22). These workers uniformly obtained the same order of change, whether prepared zymase or live cells were used. It should, of course, be kept in mind that zymase activity is not necessarily correlated with growth (22), though the original contention of Wildiers and the later work of Miss Bachmann both indicate as much. Von Euler and Karlsson (26) show that the zymase is not stimulated by biological extracts except in the presence of both coenzyme and phosphate, proving that the acceleration

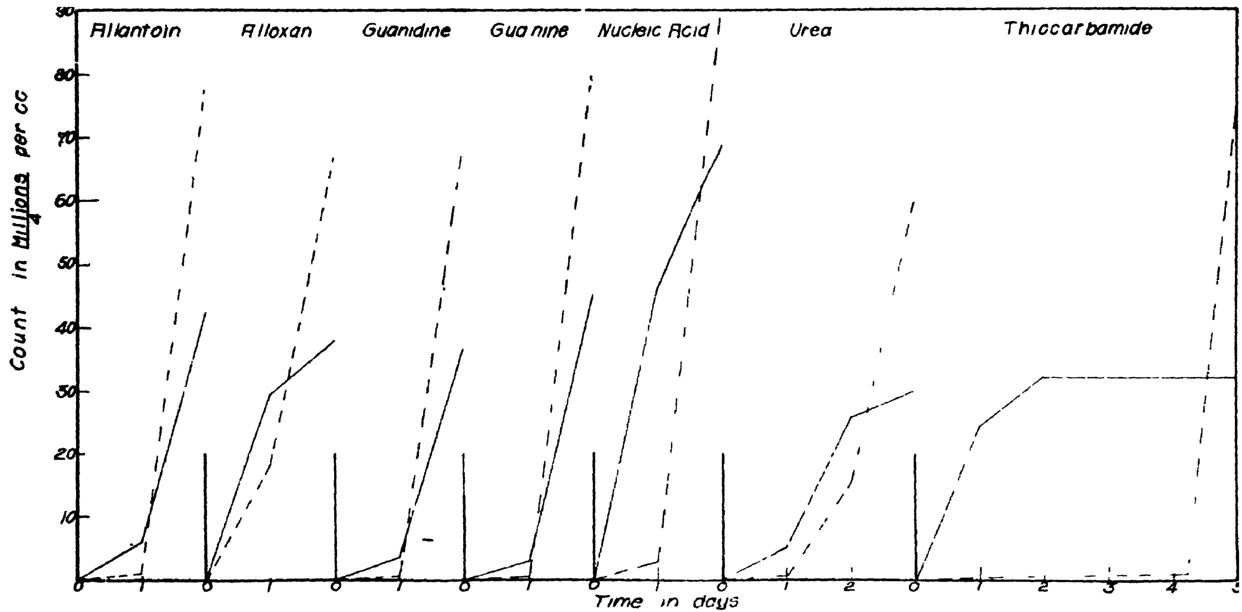

FIG. 3. Growth curves of yeast in Cultures A and B in Table V. Both cultures contain 6 drops of beer-wort. The solid line represents cultures containing only the nitrogen compound in question; the dotted line represents cultures containing nine-tenths ammonium chloride and one-tenth the nitrogen compound in question.

cannot be due to supplying a need in these two known factors. Abderhalden in a recent paper (22) points out that the slight acceleration of CO_2 production obtained through addition of known organic compounds as found by the investigators referred to above is of an entirely different magnitude than that obtained through addition of yeast juice. He concludes that the acceleration is due to as yet unknown substances.

CONCLUSIONS.

By correlating the results of other workers with the data presented in the present paper, the following conclusions concerning the bios hypothesis of yeast nutrition seem to be justified:

1. Bios is not identical with the water-soluble B vitamine, since it differs from the latter in its solubility in alcohol, resistance to alkalies, ease of adsorption onto fullers' earth, and precipitation by phosphotungstic acid and by mercuric chloride.
2. Normal growth of yeast is impossible without bios.
3. Bios is of the nature of a vitamine.
4. Up to an optimum concentration of bios, the rate of growth and the total amount of growth are roughly proportional to the amount of bios available.
5. Bios may not function directly in growth, but through assisting in the work of the cell enzymes.
6. Over 60 compounds of nitrogen, including 19 by the present writers, have been tested and found not to be bios itself.

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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

SEVENTEENTH ANNUAL MEETING.

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THE EFFECT OF ANOXEMIA ON METABOLISM.

By E. J. SCHNELLER, E. H. BRUNQUIST, and A. S. LOEVENHART.

(*From the Pharmacological Laboratory of the University of Wisconsin, Madison.*)

It has been found in our laboratory that a reduction of oxygen in the respired air to from 6 to 9 per cent results, first, in a condition of alkalosis, as has been previously found by Haggard and Henderson, and later in the production of a very marked acidosis. The extent of the acidosis depends on how much we lower the oxygen content of the atmosphere which the animal breathes.

The relation of anoxemia to acidosis is being presented elsewhere but it is necessary to give this brief résumé in order to interpret the changes in the metabolism.

Anoxemia steadily maintained from 3 to 5 days, causes: (1) an increase in the endogenous nitrogen metabolism. (2) Induces an increase in the excretion of incompletely degraded metabolites, as shown by the increased elimination of total organic acids. (3) Changes the creatine and creatinine ratio, the creatine excretion being much increased and becoming greater in many cases than the creatinine excretion.

THE URINARY SULFUR OF FASTING STEERS.

By THORNE M. CARPENTER.

(*From the Nutrition Laboratory, Carnegie Institution, Boston.*)

Two steers of about 600 kilos body weight were fasted for 5, 7, 10, and 14 days. The urines were collected every 24 hours. The inorganic sulfate, total sulfates, and total sulfur were determined in these urines by Fiske's[1] method. The maximum and minimum values for the three forms of sulfur (calculated as sulfur) were as follows: inorganic sulfate, 2.88 and 0.02 gm.; the ethereal sulfate, 4.23 and 0.05 gm.; neutral sulfur, 1.73 and 0.11 gm.

[1] **Fiske, C. H., *J. Biol. Chem.*, 1921, xlvii, 59.**

The percentage distribution ranged as follows: inorganic sulfate, 0.4 to 75.6 per cent; ethereal sulfate, 2.7 to 93.7 per cent; and neutral sulfur, 8 to 50 per cent. In general, however, the percentage of neutral sulfur was more constant than the other two. The nitrogen sulfur ratio was fairly constant in all the fasts except in the 7 day fast. Its value averaged about 20:1.

FURTHER OBSERVATIONS ON THE CHEMICAL COMPOSITION OF THE BODY FLUIDS OF THE SEA-LION.

By ROBERT E. SWAIN AND N. W. RAKESTRAW.

(From the Department of Chemistry, Stanford University, Stanford University.)

Analyses were made of the blood, pericardial fluid, milk, and urine of ten sea-lions (*Eumetopias stelleri*). The samples were taken from animals killed on the rookery on Ano Nuevo Island, off-shore from Pescadero, California. A few especially noteworthy results are the high fat content of the milk (24.8 per cent), and total solids amounting to 50 per cent. In the blood unusually high values, with wide variations, were found in total N, non-protein N, amino-acid N, and urea. A peculiar condition arose in connection with uric acid. None was found in the blood or pericardial fluid by the method of Folin-Wu, but good values were indicated by the methods of Benedict and Morris and Macleod, although these showed poor agreement, but a constant variation. A remarkable increase in the uric acid in the blood, but not in the pericardial fluid, was apparent in several cases on standing. Uric acid was found in the urine. Extraordinarily high values were obtained for total lipoids in the blood and pericardial fluid.

SYNTHESIS OF ORNITHINE IN THE FOWL.

By JAMES H. CROWDLE AND CARL P. SHERWIN.

(From the Chemical Research Laboratory, Fordham University, New York.)

Previous work has shown that the animal placed on a carbohydrate diet whereby the nitrogen metabolism was reduced to a minimum, is still able to synthesize two of the amino-acids

(glycocoll and glutamine) from endogenous waste nitrogen, which would otherwise appear as urea nitrogen. Cystine cannot, however, be synthesized under these circumstances.

In order to determine the possibility of ornithine synthesis, a hen was provided with prenatural anus which allowed the separate collection of urine and feces. The hen was kept on a non-nitrogenous diet until the endogenous urinary nitrogen excretion was constant. She was then fed benzoic acid. Both free and combined benzoic acid were determined. The combined benzoic acid was identified as the dibenzoyl ornithine. Total nitrogen, urea nitrogen, uric acid nitrogen, and ammonia nitrogen were determined.

The feeding of the acid caused a slight rise in total nitrogen, a relative decrease in uric acid nitrogen, but a decided increase in the other nitrogenous urinary excretions, which seems to indicate the synthesis of ornithine at the expense of the uric acid nitrogen. Finally, along with the benzoic acid were fed first, proline, then histidine, and lastly arginine. The latter added substantially to the output of ornithine. Proline in a less degree increased the ornithine synthesis, but histidine seemed to have little effect. All these amino-acids were readily catabolized and a greater part of their nitrogen was excreted in the uric acid fraction or ammonia fraction.

THE EFFECT OF CERTAIN NITROGEN COMPOUNDS UPON THE ACTIVITY OF UREASE.

By E. W. ROCKWOOD and W. J. HUSA.

(*From the Laboratory of Physiological Chemistry, University of Iowa, Iowa City.*)

Jack bean urease was used. The nitrogen compounds were used in 0.001 M concentration. The pH was kept at 7.5 by 0.5 M phosphate buffer solution: Fermentation continued about 2 hours at room temperature, then K_2CO_3 was added and the ammonia removed by aeration. The α-aminocarboxylic acids markedly accelerated the fermentation, the β acids much less. The greater the number of carbon atoms between the NH_2 and CO_2H group the less was the effect; increasing the length of the carbon chain also decreased the promoter effect. Amines

and acid amides had no accelerating action, nor did creatine, creatinine, or uric acid. Stereoisomeric amino-acids showed no definite difference. The carboxyl group alone or the amino group alone is inactive.

THE METABOLISM OF GALACTOSE.

I. THE THRESHOLD OF TOLERANCE IN NORMAL INDIVIDUALS.

By ALLAN WINTER ROWE.

(From the Department of Chemistry, Evans Memorial Hospital, Boston.)

Provocative galactosuria was suggested in 1906 by Bauer as a test for liver function. The fairly extensive literature is chiefly clinical in character. The "carbohydrate anomaly" is ignored and positive reactions are recorded only on appearance of relatively large amounts of sugar. With the complex control mechanism of carbohydrate assimilation and utilization, the test is susceptible of other application and possible diagnostic use suggested. Since it is necessary to establish the normal threshold, a series of studies has been made on the normal man and woman using galactose of highest purity and carrying out, on standard diet, 7 day studies of general metabolism and functional capacity with the sugar tests as the final step.

	17 women.		16 men.	
	Range	Average	Range.	Average.
Age, *yrs*	19–52	26	20–70	29
Weight, *kg*	47 0–89.1	58 8	54.8–83.2	67.8
Height, *cm*	151–181	162	164–182	172
Area, *sq. m*	1 44–1.99	1.61	1.54–1.98	1.80

The urines were tested qualitatively by Benedict's solution and on appearance of sugar, amounts were quantified.

	gm.
Average exciting dose in men	27.4
" " " " women	38.5

It is suggested that the evident sex difference rests on Folin's tissue absorption theory and potential mammary function in females. A series of blood sugar curves tend to show the character of the curve conditioned by the size of the dose. Of six anomalous curves, five were with doses below that exciting a melituria. Numerous other curves show normal rise and fall.

In pathological cases showing high threshold, 15 gm. of urea were given with the galactose test meal. Blood and urine urea curves show that the high level was not dependent upon retarded absorption. It is concluded that the dose of galactose exciting a definite melituria is of the order of 30 gm. for men and 40 gm. for women.

POSTMORTEM ACIDITY.

By ELMER L. SEVRINGHAUS.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

Autolyzing liver undergoes small changes in hydrogen ion concentration. If alkali is added to the autolyzing tissue the initial hydrogen ion concentration approaches neutrality very rapidly at first, then with a decreasing rate. This same return to neutrality is observed with acidified tissues, but the changes are not so large, and the tissues remain definitely acid. Both bases and acids must be formed during autolysis. The amphoteric amino-acids do not seem adequate to account for this buffering. A comparison of the total water-soluble acidity in such autolyzing livers, by titration with phenolphthalein, shows that the acidified tissues develop more acid than the control or the alkaline ones. These acids must be weak acids, because they appear with the hydrogen ion concentration changing rather little in the acid tissues, but markedly in the alkaline tissues. The water-soluble acids are sufficient in amount to account for 60 to 80 per cent of the buffering of the alkali. Fatty acids also play a small part, carbon dioxide less, and lactic acid probably still less.

Phosphoric acid, determined by the Bell-Doisy and the Briggs methods as inorganic phosphate, appears to make up most of the water-soluble acidity. The inorganic phosphate content of dog's liver is 15 to 20 mg. per 100 gm. of liver, in samples taken 7 to 20

minutes after the death of the animal. This amount increases rapidly at first, then more slowly to a total of 250 to 300 mg. per 100 gm. of liver after 20 days. One-third or more of the total inorganic phosphate is liberated by the end of 6 hours after death. The addition of alkali increases it. These effects are proportional to the amounts of alkali or acid.

The acidified tissues develop more total acid, but less phosphoric acid than the others, and therefore this must be due to some other weak acid. It is suggested that phosphoric acid may be a specific buffer mechanism *versus* alkali development in tissues.

HYDROGEN ION CHANGES ACCOMPANYING DEATH.

By ALFRED E. KOEHLER.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

In the study of the reaction of autolyzing liver brei it was noted that the initial pH several hours after death was in the neighborhood of 6.6. In greatly shortening the period after death it was found that the increase in acidity is not a gradual one but that a reaction of about pH 6.7 is developed in the first minutes which does not change subsequently to any marked extent. In this study dogs were killed pra tically instantaneously by shooting and the tissues removed at once, ground to a brei, and mixed with enough water or 0.8 per cent NaCl solution so as to flow into a hydrogen electrode vessel.

Similar results were obtained with muscle tissue. In an attempt to eliminate the macerating effect upon the tissue, perfusing the leg of a dog with adjusted Ringer's solution was tried. Death resulted in a rapid and marked change of the perfusate from a pH of 7.3 before to 6.8 after death.

THE RISE OF ACIDITY IN AUTOLYSIS.

By WITHROW MORSE.

(From the Department of Physiological Chemistry, School of Medicine, West Virginia University, Morgantown.)

Studies upon different organs of guinea pigs and of rabbits show a depletion of the alkali reserve of these tissues immediately

after death. The mercury pump of Van Slyke was used in the determinations, the method being followed as used for blood plasma. The organs were excised after rapid laparotomy following the killing of the animal by occipital stroke. The heart was beating during excision. The organs were placed under oil, ground in sand, centrifuged, and the layer beneath the oil was used for the determinations. The tissues (kidney, spleen, and liver) showed a degree of carbon dioxide-combining power below that exhibited by the blood plasma of a patient in diabetic coma.

THE PRODUCTION OF ACIDOSIS BY ANOXEMIA.

By A. E. KOEHLER, E. H. BRUNQUIST, AND A. S. LOEVENHART.

(From the Departments of Pharmacology and Physiological Chemistry, University of Wisconsin, Madison.)

The view that decreased oxidation in the body leads to the accumulation of acid products has been held for many years, and it has often been assumed that decreased oxidation leads to acidosis. Yandell Henderson and Haggard questioned this conclusion and demonstrated a decrease in the H ion concentration of the blood, *i.e.* alkalosis, as a result of short periods of decreased oxygen supply. They questioned whether decreased oxidation can produce acidosis. The alkalosis found by Henderson is due to the rapid excretion of CO_2 by the lungs. It was the view of the writers that decreased oxidation does lead to acidosis and their views were put to test by the following method.

Pigs were exposed to atmospheres low in oxygen with a low CO_2 content. The respiratory chamber, described by Dallwig, Kolls, and Loevenhart, was used. The electrometric determination of the pH of the blood was made together with the total CO_2. The authors confirmed the findings of Henderson and Haggard and found a very definite increase in the pH of the blood from 7.4 to the region of 7.55 in the early stages of anoxemia. On continuing the anoxemia several hours, the pH returns to normal and continues to decrease. Marked increase in the respiratory rate begins as soon as the animals are exposed to low oxygen and continues throughout the experiment until near the end. The respiratory rate rises from the normal of approximately 40 to 200

per minute. There is but a short interval of time between the fall in the respiratory rate and death. The decrease in the respiratory rate indicates exhaustion of the respiratory center. At this time, we have frequently found that the pH of the blood slows to 6.7 several minutes after the animal is removed from the chamber into the ordinary atmosphere. If the animal recovers, the return to normal is rapid—usually being complete in less than an hour—both in regard to blood pH and CO_2 content. The pH may then rise above normal (7.55). This alkalosis may last 1 hour, but the normal is finally reached. At the height of acidosis, the total CO_2 of the blood may fall to 10 per cent. The rise in CO_2 proceeds simultaneously with the rise in the blood pH.

The data prove the development of acidosis as a result of decreased oxygen supply. We believe that the production of fixed acids begins at once but does not keep pace with the loss of CO_2 from the blood, so that alkalosis is first noted. Later, the acid production more than compensates for the loss of CO_2 with the resulting very low pH values. Various phases of this work are being continued with the cooperation of Messrs. H. M. F. Behneman, O. E. Benell, and H. L. Schmitz.

THE REFRACTIVE INDEX AND WATER CONTENT OF THE BLOOD SERUM OF THYROPARATHYROIDECTOMIZED AND PARATHYROIDECTOMIZED ALBINO RATS.

By FREDERICK S HAMMETT.

(From The Wistar Institute of Anatomy and Biology, Philadelphia)

Albino rats were thyroparathyroidectomized or parathyroidectomized at 100 days of age. Determinations were made of the refractive index and water percentage of the blood serum at 150 days and compared with that of their litter controls.

It was found that thyroparathyroidectomy induced an absolute increase in the refractive index and a decrease in the water content of the serum of the male rats. The serum of the female thyroparathyroidectomized rats did not differ from that of the controls in absolute values. Parathyroidectomy did not produce alterations in the absolute values of the serum of either the males or females.

The refractive index of the serum of albino rats is a linear function of the water percentage. It is thus possible to determine whether or not a given experimental procedure has caused a change in the chemical nature of the substances (aside from water) giving rise to refraction by equating the observations for any given set of groups and comparing the slopes and the positions of the lines so obtained. The results of such an analysis show that: (1) the chemical nature of the serum of normal female rats is the same as that of the normal males; (2) thyroparathyroidectomy of male rats does not induce alterations in the chemical nature of the refracting substances. Hence the decrease in water content of the blood serum of the rats of this group is a real desiccation without chemical change. (3) Parathyroidectomy of both male and female rats causes a marked and significant change in the nature of the refractive substances. This change is in the same direction and of the same order of magnitude for both sexes. The comparison of the effect produced by the loss of the parathyroids with that produced by the loss of the thyroid apparatus clearly shows a fundamental dissimilarity in the respective metabolic upsets. (4) Thyroparathyroidectomy of the females induced an alteration in the nature of the refractive substances of the serum in the same direction as that produced by parathyroidectomy. The difference in response of the females from that of the males which were thyroparathyroidectomized accompanied a much more marked growth retardation in the former. In fact, the female thyroparathyroidectomized rats lost weight immediately after the removal of the glands and never regained their initial status. This fact is sufficient to account for the change in the nature of the refractive substances in this group as compared with the males of the same group and the controls.

THE RELATION OF FRACTURE HEALING TO THE INORGANIC PHOSPHORUS OF THE BLOOD SERUM.

By WALTER H. EDDY AND HATTIE L. HEFT.

(From the Department of Pathology, New York Hospital, and the Department of Physiological Chemistry, Teachers College, Columbia University, New York.)

Tisdall and Harris have recently presented data[2] which suggest a direct relation between the inorganic phosphorus content of the blood serum and the progress of fracture healing. Their article stimulated the series of studies which we have begun with the cooperation of Drs. C. R. Murray and Ralph Stillman of the New York Hospital. In this preliminary report we wish to present some of our findings to date.

The blood of twenty-three fracture cases has been examined to date, several of them repeatedly. Practically all these cases show an abnormally high phosphorus and the progress of healing bears a definite relation to the amount of phosphorus found. One non-union case which showed a normal amount 40 days after fracture promptly developed a rise following operative reduction and the use of a caliper splint. These findings confirm those of Tisdall and Harris.

We have also, however, examined the bloods of some twenty cases involving surgical operation and whose recovery involved the healing of tissue other than bone. The same high phosphorus is found in these cases also. CO_2 capacity determinations were made on all the cases examined and the results cannot be laid to variations in this factor. This second result suggests that we must at least proceed cautiously before accepting the phosphorus findings as a specific index of bone formation and the desirability of more detailed inquiry into what makes the increase.

Dr. Murray suggested that it was a common belief that syphilis is an inhibiting influence in fracture healing. Examination of the bloods of some thirty positive Wassermann cases fails to show abnormal phosphorus findings in these cases. In regard to the normal value, however, we take exception to Tisdall and Harris' curve. Our studies of normal bloods of persons between the

[2] Tisdall, F. F., and Harris, R. I , *J. Am Med. Assn.*, 1922, lxxix, 884.

ages of 15 and 65 indicate that 2.9 mg. of phosphorus per 100 cc. of serum is nearer the normal figure.

Finally, we wish to add that the above determinations have been made by the Tisdall method. We have checked this method repeatedly against the Briggs modification of the Bell-Doisy method and find that the two methods give equally accurate results, but we find the greens of the Tisdall method present less difficulty in matching colors.

THE PHOSPHORUS AND CALCIUM CONCENTRATION OF THE SERUM OF PATIENTS DURING THE PERIOD OF FRACTURE UNION.

By J. J. MOORHEAD, H. W. SCHMITZ,* LOIS CUTTER, AND V. C. MYERS.

(From the Departments of Traumatic Surgery and Biochemistry, New York Post-Graduate Medical School and Hospital, New York.)

In 1920 a few calcium estimations were made on fracture cases with non-union, also cases with very rapid bone regeneration, but these analyses did not appear to disclose any abnormality in the calcium content of the serum.[3] Our interest in this problem has again been aroused by the important observation of Tisdall and Harris,[4] that during the period of union of fractures in adults, the phosphate content of the serum is raised to a level approximately the same as that present in childhood. The present preliminary study includes observations on about ninety cases, thirty of which were fracture cases. The method of Briggs[5] was employed for the inorganic phosphorus and that of Clark[6] for the calcium.

The average inorganic phosphorus found in thirty normal adults was 3.7 mg. per 100 cc., while in ten children under 10 years it was slightly below 5.0 mg. The low average observed in

* Medical Fellow of the National Research Council.

[3] Myers, V. C., Chapter on body tissues and fluids, in Barker, L. F., Endocrinology and metabolism, New York, 1922, iii, 451.

[4] Tisdall, F. F., and Harris, R. I., *J. Am. Med. Assn.*, 1922, lxxix, 884.

[5] Briggs, A. P., *J. Biol. Chem.*, 1922, liii, 13.

[6] Clark, G. W., *J. Biol. Chem.*, 1921, xlix, 487.

children is probably to be explained by the fact that some of the cases were undernourished.

The inorganic phosphorus of the blood serum has generally been found to rise following fracture, but in our series 5.0 mg. or more have been attained in rather less than half the cases. The highest values are sometimes observed in 2 or 3 days after the fracture, at other times not until a month has elapsed. In one case union was observed, although there was only a slight rise in the phosphorus, 2.5 to 3.2 mg. In another case there was non-union, although the phosphorus reached 5.0 mg. after 3 days and did not drop to 3.8 until a month later. In a series of six fracture cases in children phosphorus values of 5 mg. were observed in only one case, although in the four cases having more than one observation all showed a rise. In two cases with mal- and non-union there was a rise in the phosphorus following operation. In two cases of non-union, however, there was a slight drop following operation. One of these still shows non-union at the end of 2 months. A series of nine cases with minor fractures (fingers) discloses negative results. Several of the cases appear to show slight variations in the calcium, paralleling the phosphorus.

OBSERVATIONS ON THE NATURE OF THE CARBOHYDRATE OF NORMAL URINE.

By ISIDOR GREENWALD.

(*From the Harriman Research Laboratory, Roosevelt Hospital, New York.*)

The excretion of sugar in the urine of a man and of a dog was as high on a protein-fat diet furnishing an adequate amount of energy as it was on a carbohydrate diet. The 24 hour quantity was not increased in the dog, by the ingestion of as much as 10 gm. of glucose per kilo, when the animal was on a carbohydrate diet. When the same quantity was given with a protein-fat diet, there was a slight increase in the quantity of sugar in the urine, the increase being the greater, the larger the amount of fat in the diet. The man showed an apparently perfect tolerance for 1 gm. of glucose per kilo body weight when on a diet containing about 50 gm. of fat and furnishing about 11 gm. of nitrogen in the urine. When the fat was increased to about 140 gm. per day, there was

a slight increase in the amount of sugar in the urine. On the protein-fat diet, about 0.05 gm. of glucose per kilo body weight appeared in the urine. The excretion of sugar was not affected by the ingestion of 12 gm. of thymus, or yeast nucleic acid, by the man.

Several osazones were obtained from mixed normal human urine. One of these was identified as lactosazone. Another is probably pentosazone, for pentose was demonstrated in other ways to constitute about one-third of the total sugar. An osazone insoluble in hot water and melting at about 200° was also obtained, but under the microscope appeared to be a mixture.

It is suggested that pentose may be formed from protein and that pentosuria is a disorder of protein, not of carbohydrate, metabolism.

A STUDY OF THE NON-CYSTINE-PROTEIN SULFUR.

By J. HOWARD MUELLER.

(From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York, and the Biochemical Laboratory, Cambridge University, England.)

Some time ago the writer reported at the Society of Experimental Biology and Medicine in New York, the isolation of crystalline material from a sulfuric acid hydrolysate of casein. After repeated recrystallization, this material had the composition represented by the formula $C_{11}H_{23}SN_2O_4$. Further work has shown that it contained considerable phenylalanine and at least one other unidentified amino-acid in addition to the sulfur compound. It has now been possible by two quite different methods to isolate the sulfur compound in a much purer form, corresponding quite closely to the formula $C_5H_{11}SNO_2$. It has been obtained after both sulfuric acid and sodium hydroxide hydrolysis of casein in about the same yield, and also from several other proteins. The sulfur is not in the lead blackening form, and does not give a red color with sodium nitroprusside in the form isolated, nor after reduction. The nitrogen is split off quantitatively by nitrous acid, indicating an α-NH_2 group; CO_2 is given off on heating and salts are formed with metals, and a COOH group, therefore, appears to be present.

Fusion with NaOH, and heating in a sealed tube produce a volatile sulfur compound giving a red color with sodium nitroprusside. The structure is being further investigated.

THE SEPARATION OF THE HEXONE BASES FROM CERTAIN PROTEIN HYDROLYSATES BY ELECTROLYSIS.

By CARL L. A. SCHMIDT and G. L. FOSTER.

(From the Department of Biochemistry and Pharmacology, University of California, Berkeley.)

Experiments have been carried out with hydrolyzed gelatin, casein, and fibrin, which show that the hexone bases can be separated from the other amino-acids by electrolysis. The hydrolysate is placed in the center of a three-compartment cell and distilled water is placed in each of the end compartments. These are separated by gelatin membranes and carbon electrodes are used. On passing a current through the cell the hexone bases wander to the cathode. Glutamic and aspartic acid and the pigment migrate to the anode, while the other amino-acids remain in the center compartment. When the pH of the center compartment is kept at 7.5 histidine does not wander to the cathode but at pH 5.5 the three hexone bases migrate to the cathode in about the same ratio as they existed in the protein.

IODINE IN NATURAL WATERS IN RELATION TO GOITER.

By J. F. McCLENDON.

(From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.)

Kendall found that foodstuffs lose iodine in charring, but I burn them in a calorimeter-bomb to prevent loss. Foodstuffs derive iodine from soils, and river water represents leachings from soils. In the Mississippi river system, in Minnesota, iodine averages 0.8 (per billion) and goiter 8 (per 1,000 troops); in Missouri, iodine is increased to 2 and goiter reduced to 4.

The distribution of Cl and I might show similarity because of similar solubility. The United States is divided into four regions: first, goiter 15 to 30 (per 1,000 troops) and Cl 0.5 to 2 (per cent

of total dissolved solids in representative rivers); second, goiter 5 to 15 and Cl 1 to 3; third, goiter 1 to 5 and Cl 2 to 5; fourth, goiter 0 to 1 and Cl 4 to 30.

In the iodine determination, 100 liters of water are made alkaline, evaporated to dryness, and charred in the bomb with threads, water-cooled to prevent welding. The char is leached with boiling water and iodate and iodide changed to iodine and determined in CCl_4 with a Bausch and Lomb colorimeter with a cup holding 1 cc.

Rats fed KI by myself for two generations had thyroids averaging half the weight of those of Dr. C. M. Jackson's rats fed no iodine.

THE EFFECT OF SOME ORGANIC ACIDS ON THE EXCRETION OF URIC ACID BY MAN.

By HARRY V. GIBSON and EDWARD A. DOISY.

(*From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.*)

In an attempt to analyze the effect of amino-acids upon the excretion of uric acid[7] possible deamination products were ingested by two normal men. Pyruvic acid produces a rise while lactic acid causes a very sharp decrease in the hourly excretion of uric acid.

The effect of lactic acid suggests a partial explanation of the curve of excretion of uric acid[8] during and following exercise. The lactic acid produced may well account for the decreased excretion of uric acid during exercise, but *per se* probably does not explain the increased purine elimination.

[7] Lewis, H. B., Dunn, M. S., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 9.

[8] Kennaway, E. L., *J. Physiol.*, 1909, xxxviii, 1.

A CHEMICAL STUDY OF SALIVA.

By J. LUCIEN MORRIS and VERNON JERSEY.

(From the Biochemistry Department of the Western Reserve University School of Medicine, Cleveland.)

Saliva from adults and children was analyzed. The secretion was collected from the quiescent glands during fasting and varied diets. Observation was made of the effect of mechanical and chemical stimuli.

Increase of cellular activity took place as the day progressed. Urea, uric acid, creatinine, and amino-acids in the secretion were readily determined by microchemical methods. Reducing sugar was not present in normal saliva. Urea and amino-acids showed variations proportional to volume changes. Uric acid and creatinine were more constant and relatively more independent of the volume.

EFFECT OF FOOD INGESTION ON THE CLOTTING TIME OF THE BLOOD.

By C. A. MILLS.

(From the Department of Biochemistry, College of Medicine, University of Cincinnati, Cincinnati.)

The clotting time of the blood is longest before breakfast in the morning, but shows only slight variations at this time on successive days. It shortens 30 to 40 per cent about an hour after each meal and 2 to 3 hours later begins to lengthen. 5 or 6 hours after each meal it will be almost at the same point as on rising in the morning. This clotting time observed before each meal is termed the "basal clotting time."

The cause of this effect of food ingestion remains in obscurity. The clotting time bears no relation to the specific gravity of the blood so far as physiological variations go. Water taken produces no change in the clotting rate; neither does glucose solution nor eating plain sugar candy. Coagulated egg white produces no change for about an hour after eating, but later causes the typical increased coagulability of the blood. The effect on the blood then, seems to depend on the presence of the food in the intestine.

Whether it is the absorbed material that causes the change cannot be said. Acid or alkali ingestion sufficient to alter markedly the reaction of the urine produces no change in the clotting time.

THE INFLUENCE OF CARBON DIOXIDE ON THE VELOCITY OF SEGMENTATION OF SEA URCHIN EGGS.

By G. H. A. CLOWES and HOMER A. SMITH.

(*From the Biochemical Research Laboratory, Eli Lilly and Company, Indianapolis.*)

Methods have been devised whereby the influence of CO_2 on the segmentation of sea urchin eggs and larvæ could be studied in a quantitative manner. It has been found that the velocity of segmentation is an *inverse linear function* of the concentration of CO_2. This function is independent of the concentration of hydrogen, sodium, or calcium ions in the sea water (within those limits, of course, which are compatible with normal development), and it is apparently unaltered by variations in temperature between 15 and 30°C. CO_2 causes a progressive retardation, or, at definite tensions, complete arrest of development at all stages from the freshly fertilized egg to the larval stage without causing any alteration in normal cell structure (cytolysis, abnormal division, etc.) such as are caused by most deleterious agents.

A GAS ANALYSIS APPARATUS FOR USE WITH CHAMBER RESPIRATION APPARATUS.

A DEMONSTRATION.

By THORNE M. CARPENTER.

(*From the Nutrition Laboratory, Carnegie Institution, Boston.*)

The gas analysis apparatus is constructed on the Haldane principle. The graduation of the burette is so designed that a carbon dioxide content up to 1.700 per cent and an oxygen deficit up to 2.000 per cent can be determined. From the tap to the first graduation is a bulb with a capacity of 31.36 cc. From 31.36 to 31.84 cc. there is a capillary tube with divisions of 0.004 cc. From 31.84 to 39.36 cc. is a second bulb and from 39.36 to 40.04 cc. there is another capillary tube divided so that each division cor-

responds to 0.004 cc. The burette is marked in percentage and the smallest division corresponds to 0.01 per cent and estimations may be made to 0.001 per cent. The pyrogallic acid container is made after the pattern of Krogh's apparatus.[9] The following series of analyses of outdoor air and analyses of air from a chamber with an ethyl alcohol flame in it illustrate the results obtainable with the apparatus.

Analyses of outdoor air.		Analyses of air from an ethyl alcohol flame.		
Analyst: E. L. F., Sept. 26, 1922.		Analyst: M. L. B., July 22, 1922.		
CO_2	O_2	CO_2	O_2 deficit.	R. Q.*
per cent	*per cent*	*per cent*	*per cent*	*per cent*
0.027	20.939	0.638	0.970	0.658
0.031	20.942	0.665	1.002	0.664
0.029	20.944	0.664	1.009	0.660
0.034	20.940	Analyst: L. A. R., Oct. 12, 1922.		
0.030	20.939			
		0.551	0.845	0.652
		0.512	0.773	0.662
		0.358	0.532	0.673

* Theoretical R. Q. = 0.667.

THE ESTIMATION OF BILE ACIDS IN ICTERUS URINE.

By CARL L. A. SCHMIDT and JOHN A. MERRILL.

(*From the Department of Biochemistry and Pharmacology, University of California, Berkeley.*)

The procedure is as follows: The urine is evaporated at a low temperature, the bile acids are dissolved in absolute alcohol, the alcohol is evaporated, and the bile acids are taken up in a small quantity of water and precipitated in the cold by saturation with $MgSO_4$. The precipitate is washed with a saturated solution of $MgSO_4$ and then dissolved in absolute alcohol. The alcohol is evaporated and the amino nitrogen is estimated in separate portions of the aqueous solution before and after hydrolysis with 8 per cent NaOH. The difference gives the nitrogen of the bile acids.

[9] Krogh, A., *Biochem J.*, 1920, xiv, 269.

THE BENZOATE TEST FOR RENAL FUNCTION.

By F. B. Kingsbury.

(*From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.*)

44 tests have been made on normal individuals and 39 on patients with renal disorders. A few comparisons have been made with the urea concentration test of MacLean,[10] the water excretion test of Volhard,[11] the constant Austin, Stillman, and Van Slyke,[12] and the phenolsulfonephthalein test of Rowntree and Geraghty.[13] A fairly close agreement has been found between the findings by the benzoate test and by the others with the exception of the phenolsulfonephthalein test in cases of hypertension and the nephrosis due to the toxemia of pregnancy. In well marked nephritis the benzoate test is always low and the findings agree with the blood urea and phenolsulfonephthalein criteria. The benzoate test indicates impairment of renal function earlier than does the retention of urea in the blood.

The benzoate tests were made uniformly as follows: The subject voids urine and immediately thereafter drinks a solution of 2.4 gm. of sodium benzoate in 100 cc. of water. The container is rinsed with about 200 cc. of water and this also is drunk. At the end of 1 hour 200 cc. of water are drunk. 2 hours after ingesting the sodium benzoate the subject collects the first specimen of urine. 1 hour later the second sample of urine is collected. The test is made before breakfast and all food avoided until after the test. The urine samples are separately analyzed for hippuric acid by the method of Kingsbury and Swanson.[14]

The total excretion of hippuric acid in the first 2 hours has been shown normally to be at least 70 per cent of that theoreti-

[10] MacLean, H., and de Wesselow, O. L. V., *Brit. J. Exp. Path.*, 1920, i, 53.

[11] Volhard, F., and Fahr, T., Die Brightsche Nierenkrankheit Klinik, Pathologie und Atlas, Berlin, 1914, 121 and 126.

[12] Austin, J. H., Stillman, E., and Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlvi, 91.

[13] Rowntree, L. G., and Geraghty, J. T., *J. Pharmacol. and Exp. Therap.*, 1909–10, i, 579.

[14] Kingsbury, F. B., and Swanson, W. W., *J. Biol. Chem.*, 1921, xlviii, 13.

cally obtainable from 2.4 gm. of sodium benzoate and the total for 3 hours to be at least 90 per cent.

The findings in this report confirm those of Kingsbury and Swanson[15] and extend the usefulness of this test.

My thanks are due to Dr. George E. Fahr of the Department of Medicine for helpful criticism and for his cooperation in furnishing data of the other renal tests mentioned.

THE SYNTHESIS AND RATE OF ELIMINATION OF HIPPURIC ACID IN THE ORGANISM OF THE RABBIT.

By HOWARD B. LEWIS and WENDELL H. GRIFFITH.

(*From the Laboratories of Physiological Chemistry of the University of Illinois, Urbana, and of the University of Michigan, Ann Arbor.*)

Previous studies on the precursors of the glycocoll made available for the detoxication of benzoic acid have been unsatisfactory, inasmuch as the rabbit, the experimental animal usually employed, can conjugate almost completely any non-lethal dose of benzoate in a period of 24 hours, regardless of the presence of possible precursors of glycocoll. It seemed probable that in the presence of a metabolite which could form glycocoll the velocity of the formation and elimination of hippuric acid after benzoate ingestion might be increased, and accordingly, studies of the rate of elimination of hippuric acid over short periods of time have been made in rabbits. Hippuric acid administered intravenously was eliminated almost quantitatively in 6 hours, while after intravenous injection of an equivalent quantity of sodium benzoate, less than 50 per cent was eliminated as hippuric acid in a like period. The rate of elimination of hippuric acid after enteral ingestion of sodium benzoate was not markedly different from that obtained after intravenous injection. However, when glycocoll was administered with sodium benzoate *per os*, the rate of hippuric acid elimination was greatly increased. This increase in the rate of elimination might be considered to be due to (1) a stimulation of general metabolism as a result of the specific dynamic action of amino-acids, or (2) to an increase in the rate of absorption of

[15] Kingsbury, F. B., and Swanson, W. W., *J. Biol. Chem.*, 1921, xlvi, p. iv; *Arch. Int. Med.*, 1921, xxviii, 220.

sodium benzoate from the intestine under the influence of glycocoll, or (3) to an increased rate of synthesis of hippuric acid due to the presence of abnormally large quantities of glycocoll in the organism. Since alanine fed with the benzoate did not increase the rate of hippuric acid elimination, the effect is not considered to be due to a stimulation of general metabolism. Since the rate of elimination of hippuric acid was accelerated when sodium benzoate was injected *intravenously* and the glycocoll *fed*, the results can hardly be explained on the basis of an increased velocity of absorption of the benzoate from the intestine. It seems probable that an increase in the rate of synthesis has resulted from the presence in the system of extra glycocoll or its metabolites and that such glycocoll precursors are not furnished by alanine.

THE AMINO-ACID NITROGEN IN THE BLOOD AND ITS POSSIBLE RELATION TO THE ELEVATION OF THE METABOLISM IN MYELOGENOUS LEUCEMIA.

By K. SANDIFORD, W. M. BOOTHBY, AND H. Z. GIFFIN.

(*From the Metabolism Laboratory, Mayo Clinic and Mayo Foundation, University of Minnesota, Rochester.*)

In a series of five cases of myelogenous leucemia total and basal metabolism experiments were carried out in conjunction with the study of the chemistry and morphology of the blood. The main point developed by this study was the frequent finding of an increase in the amino-acid partition of the non-protein nitrogen of the blood (Folin method), accompanied by an increase in the basal metabolism. The values ranged between 5 and 16 mg. and the average was 10 mg. (normal 5 to 7 mg.); the basal metabolic rate varied between +6 and +81 per cent. In all instances in which the amino-acids were increased there was some, but not necessarily a proportional, increase in the basal metabolism. On the other hand, as shown by four other cases in which only one determination was obtained the basal metabolism was distinctly elevated in two cases without an increase in the amino-acids. In three cases of lymphatic leucemia the amino-acids were 5, 6, and 9 mg. with a basal metabolic rate of +55, +3, and +25 per cent. In a case of polycythemia the amino-acids were 11 mg. and the

basal metabolic rate was +21 per cent. Twenty-one patients with exophthalmic goiter and six with adenomatous goiter with hyperthyroidism showed no elevation of the amino-acids or other non-protein nitrogenous substances in the blood.

THE ANTIKETOGENIC ACTION OF GLUCOSE.

By GEORGE ERIC SIMPSON.

(From the Biochemical Laboratory, Washington University School of Medicine, St. Louis.)

The urinary excretion of acetone bodies is determined for short intervals after the ingestion, by normal starving men, of (*a*) glucose alone, (*b*) hydroxybutyric acid alone, and (*c*) glucose and hydroxybutyric acid simultaneously. The antiketogenic effect of small quantities of glucose is at its height within a few hours after ingestion. The type of experiment exemplifies the procedure developed for a resurvey, now under way, of substances thought to possess antiketogenic action.

EXPERIMENTS ON THE UTILIZATION OF THE CALCIUM OF ALMONDS BY MAN.

By MARY SWARTZ ROSE and GRACE MacLEOD.

(From the Department of Nutrition, Teachers College, Columbia University, New York.)

Calcium balance experiments have been conducted on healthy young women taking for five consecutive 3 day periods a simple mixed diet in which blanched and finely ground almonds contributed from 73 to 86 per cent of the total calcium. The results, calculated for 1 kilo of body weight and averaged for the last 12 days in order to exclude fully the effect of previous diets, are shown in Table I.

With two exceptions the subjects were practically in calcium equilibrium. In these two the intake was probably too low to meet requirement. Three experiments were also conducted on a diet in which from 69 to 75 per cent of the calcium was derived from milk. The returns in terms of body weight were as given in Table II.

In Cases 1, 2, and 3 the calcium of almonds seems to have been utilized quite as well as the calcium of milk, five milk experiments (including the two previously reported) showing equilibrium on about 6 mg. per kilo. In the other almond experiments from 8 to 12 mg. appear to have been required to establish equilibrium. From these experiments the conclusion is reached that the calcium of almonds is not quite so effectively utilized as that of milk. Digestion experiments showed good utilization of the almond diet.

TABLE I.

Average Daily Intake and Output of Calcium on Almond Diet.

No.	Subject.	Body weight.	Ca from almonds.	Intake.	Output.	Balance.
		kg.	*per cent*	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*
1	B. H.	45	74	4.1	5.0	−0.9
2	M. F.	60	73	4.1	6.0	−1.5
3	M. K.	60	73	4.1	4.9	−0.8
4	D. H.	75.8	73	8.8	7.7	+1.1
5	E. B.	55	85	9.4	9.4	
6	H. G.	55	85	9.3	11.6	−2.3
7	J. R.	58	85	9.3	11.0	−1.8
8	B. B.	52.7	86	5.5	10.0	−4.5
9	H. C.	50 5	86	5.7	7.1	−1 4

TABLE II.

Average Daily Intake and Output of Calcium on Milk Diet.

No.	Subject.	Body weight.	Ca from milk.	Intake.	Output	Balance.
		kg.	*per cent*	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*
10	B. B.	54	70	6.0	6.4	−0.4
11	G. C.	62.5	74	6.4	6 2	+0.2
12	C. M.	45	71	6.6	7.4	−0.8

THE UTILIZATION OF SUGAR BY RATS DEPRIVED OF VITAMINE B.

By H. A. MATTILL.

(*From the Department of Vital Economics, University of Rochester, New York.*)

A single rat under a bell jar of 5 to 6 liters capacity produces during 10 to 15 minutes sufficient change (0.75 to 2 per cent) in O_2 and CO_2 content of the air to give satisfactory respiratory

quotients by means of the Haldane apparatus. Air is withdrawn over mercury through a tube in the top of the bell jar. The stopper also carries a thermometer and a small mercury manometer, but the data on total heat production show wide variations due to the movements of the animal. The accuracy of the respiratory quotients, however, is satisfactory. Alcohol checks can be run in 1 to 2 minutes, depending on the size of the flame employed.

The respiratory quotients on a synthetic ration containing casein, starch, lard, salts, butter fat, and yeast varied from 0.80 to 0.90. Basal quotients usually showed less variation and averaged 0.75 to 0.76. On the synthetic ration without yeast, the quotients were usually lower than those of the controls, and approached the basal values; this was to be expected as a result of the decreased food consumption. Ingestion of cane-sugar or glucose was followed by a rise in the quotient to 0.90 and beyond in both sets of animals, thus indicating that deprivation of vitamine B does not interfere with the processes of glucose combustion.

STABILITY OF VITAMINE IN COD LIVER OIL.

By H. STEENBOCK, J. H. JONES, and E. B. HART.

(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison.*)

Puppies at an age of 6 weeks on a ration of cooked white cornmeal and oat meal *ad libitum* and 5 gm. of casein, 2 gm. of sodium chloride, 5 gm. of calcium phosphate, and 200 cc. of skimmed milk per day were given in addition 5 cc. of cod liver oil or the unsaponifiable material obtained from 5 gm. of cod liver oil daily. Such dogs showed normal growth, normal Ca and P in the blood serum, normal ash content of the bones, and normal distribution of inorganic materials in bone as determined by x-ray examination.

Without the cod liver oil adjuvant, saponified or not, growth was inhibited, tetany resulted, Ca and P in serum were much reduced, and bones were poorly calcified as shown by ash content and x-ray examination.

THE NEPHROPATHIC ACTION OF MUCIC ACID.

By WILLIAM C. ROSE AND PAULINE S. DIMMITT.

(*From the Laboratories of Physiological Chemistry of the University of Texas, Galveston, and of the University of Illinois, Urbana.*)

In continuation of studies reported a year ago[16] on the nephropathic action of dicarboxylic acids, recent experiments indicate that mucic acid, when administered subcutaneously as the sodium salt in doses of 0.5 gm. or more, produces in rabbits a severe nephritis. Marked retention of non-protein nitrogen, urea, and creatinine occurs, while the phenolsulfonephthalein elimination may fall almost to zero. Microscopic examination of the kidneys shows that the lesions involve the tubules primarily, with more or less secondary involvement of the glomeruli. In some cases hemorrhagic areas may be observed in the cortex.

Adipic acid, on the other hand, is only slightly nephropathic. These data, together with those formerly reported, seem to indicate that the introduction of hydroxyl groups in dicarboxylic acids greatly increases their renal toxicity. Both tartaric and mucic acids are much more nephropathic than are the corresponding succinic and adipic acids.

Preliminary experiments on the oral administration of mucic acid indicate that, as in the case of tartaric acid, very large doses are necessary to produce nephritis in rabbits. We are extending these studies to other acids.

THE ENZYMES OF THE RED BLOOD CORPUSCLES OF THE MAMMAL.

By WITHROW MORSE.

(*From the Department of Physiological Chemistry, School of Medicine, West Virginia University, Morgantown.*)

We have previously reported that erythrocytes of the mammal exhibited no autolysis even after long periods. We wish to extend these observations to include other enzymes. The blood of the dog in addition to the absence of proteoclastic enzyme capable of digesting the tissues of the corpuscles containing hemoglobin, shows absence of amylase, esterase, and invertase, but positive findings were obtained for lipase and catalase.

[16] Rose, W. C., *J. Biol. Chem.*, 1922, l, p. xxiii.

With a view to determining the rôle which the presence of a nucleus may have in regard to the compliment of enzymes in erythrocytes, we studied the enzyme content of the blood of *Cryptobranchus alleghaniensis*, a salamander having nucleated corpuscles, but we were unable to detect any difference in suite of enzymes from that of the enucleated types of the mammal, catalase and lipase being found as in the blood of the dog.

STUDIES ON THE METABOLISM IN PREGNANCY.

I. CHANGES IN THE TENSION OF ALVEOLAR CARBON DIOXIDE.

By ALLAN WINTER ROWE.

(*From the Department of Chemistry, Evans Memorial Hospital, Boston.*)

A preliminary report of one phase of study of gaseous metabolism in pregnancy; two groups of cases studied, one from a prenatal out-patient service of a large metropolitan hospital, the other from a nursing home for unmarried mothers.

The Marriott and Fredericia methods were compared and the latter was selected as more convenient and less liable to error. Duplication of the respiratory phase was shown to be possible with the variation of ± 1.3 mm. Fredericia and Haldane were compared, using a Fredericia apparatus with a 3-way intake valve and capillary delivery as collection apparatus. Displacements were made over mercury. Deviations were less than ± 2 mm. in twenty-one series with a mean of − 0.4 mm. Haldane and Fredericia were compared in the same sample of air, collecting in the modified Plesch-Higgins collecting bag and passing air for Haldane through the modified Fredericia apparatus. Deviations in fifteen series were the same as in the preceding group with an average of ± 0.

In 56 cases of normal pregnancy carried to term with 310 observations, the average tension of alveolar carbon dioxide was 32.6 mm. In 13 cases with 45 observations of ketosis the average was 30.9 mm. In 14 cases with 61 observations of grave pathological states without ketosis the average was 32.8.

In 22 normal cases with 135 observations, the total acid, as estimated from the ammonia and titratable acid elimination, was 790 cc., an entirely normal figure. In 18 normal cases, the

average ammonia output was 0.54 gm.; the phosphoric anhydride, 1.88; and the alveolar carbon dioxide, 29.9 mm. In another series of 10 cases the figures were 0.45, 1.75, and 33.7 mm.

The ammonia is normal, the phosphates show the possibility of slight phosphate retention, the alveolar carbon dioxide is definitely subnormal. There is no certain evidence of acid retention. Vital capacity measurements show no diminution, and in many cases an increase during pregnancy. Indirect methods had to be used due to the impossibility of obtaining blood samples.

To conclude, evidence of a marked lowering of the tension of alveolar carbon dioxide in normal pregnancy is presented comparable to that observed in well defined ketosis.

SOME CHEMICAL REACTIONS OF INSULIN.

By C. H. BEST and J. J. R. MACLEOD.

(From the Department of Physiology, University of Toronto, Toronto, Canada.)

Every sample of insulin prepared by the usual method from the pancreas of the ox or pig that has been examined by us, has given a decidedly purple color by the biuret test, an orange color by the xanthoproteic test, and a faint purple (masked by browning) by the Hopkins-Cole reaction. The intensity of the reactions has varied considerably with different preparations. On the other hand, insulin prepared from the pancreas of the skate by alcoholic extraction and heat gives none of the color reactions for protein. That the trace of protein in ox insulin preparations is an impurity is evidenced by the fact that after precipitation with phosphotungstic acid and removal of the latter by ether and barium, the preparation retains its powers to cause hypoglycemia.

Insulin gives none of the usual color reactions for epinephrine.

Moderate heat does not appreciably affect the strength of insulin, at least in faintly acid reaction (pH between 5 and 6). Thus, it may be heated on a boiling water bath for at least 10 minutes, and probably much longer, without perceptible alteration in strength, but it is destroyed by actual boiling for 3 minutes. Acidified hot water may therefore be used for the extraction of insulin from the pancreas.

Moderate degrees of alkalinity (to litmus) do not affect the strength of insulin during a period of 6 minutes at room tempera-

ture. We cannot as yet state the degree of alkalinity which insulin can withstand.

Insulin in acid reaction is adsorbed by kaolin, charcoal, etc. Thus, after shaking 4 cc. of insulin with kaolin for 10 minutes, 0.8 cc. lowered the blood sugar from 0.104 to 0.060 in 1 hour, without symptoms, whereas 0.8 cc. of the original insulin lowered it from 0.098 to 0.037 in 1 hour, and caused convulsions.

It has so far been impossible to demonstrate any dialysis of insulin through collodion sacs, and usually there is also a marked reduction in the strength of insulin within the dialyser. These experiments have been conducted either by frequently changing the water outside the dialyser or by using a small volume of unchanged water. Dialysis has been allowed to proceed for 3 hours.

On several occasions it has been found that the potency of the insulin preparations is greatly reduced by passing through a Berkefeld filter. Thus:

1 cc. of ox insulin before being passed through a Berkefeld filter lowered the blood sugar in 2 hours from 0.126 to 0.042, causing convulsions, and after it, from 0.130 to 0.072 with no symptoms.

3 cc. of skate insulin before being passed through a Berkefeld filter lowered the blood sugar in 2 hours from 0.120 to 0.054, causing convulsions, and after it from 0.122 to 0.090, with no symptoms.

By the method of formol titrations (Sörensen) it has been found that proteolytic enzymes are present in extracts of ox pancreas after the removal of the alcohol by the method described elsewhere, but none has been detected in the final preparations of insulin.

THE INFLUENCE OF PANCREATIC PERFUSATES ON THE BLOOD SUGAR, D:N RATIO, AND RESPIRATORY QUOTIENT OF DEPANCREATIZED ANIMALS.

By HARRY D. CLOUGH, ARTHUR M. STOKES, C. B. F. GIBBS, NEIL C. STONE, AND JOHN R. MURLIN.

(*From the Physiological Laboratory, University of Rochester, Rochester.*)

Perfusion, by Clarke's method, of the pancreas of the dog, pig, and cow extracts the antidiabetic substance in sufficient concen-

tration to affect favorably all the major symptoms of diabetes produced by depancreatization. In several instances the blood sugar was reduced to normal or lower by a single injection; the excretion of sugar is stopped by two or three injections; and the respiratory quotient may be raised at will by repeated injections. When the perfusion fluid is made slightly acid more potent extracts are obtained. The name *glucopyron* is suggested as the one most appropriate for the active antidiabetic substance however obtained from the pancreas. There is no evidence that it is a *chalone* in Schafer's sense: hence the name *insulin* suggested by him is inappropriate.

SOME PROPERTIES OF AN ACTIVE CONSTITUENT OF PANCREAS (INSULIN).

By E. A. DOISY, MICHAEL SOMOGYI, and P. A. SHAFFER.

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.)

Insulin has been prepared from beef pancreas by modifications of methods outlined by Banting and Best and by Collip. More than fifty different preparations have been made and used in work with dogs and rabbits, in *in vitro* experiments, and with human diabetics, with marked success.

The active material has been purified and concentrated to the point that about 0.25 mg. of substance produces marked hypoglycemia and characteristic convulsions in rabbits weighing about 1 kilo. This material is a white powder having the following properties. It contains about 14 per cent nitrogen, and is free from phosphorus; it gives distinct biuret reaction, faint reaction with glyoxylic acid, and doubtful Millon's reaction. Its solutions are levo-rotary. Its activity in large part withstands boiling in 0.1 N HCl for 5 minutes. It is freely soluble in water except at C_H about 5 to 6, at which reaction it is precipitated (incompletely) from its solutions. It is soluble also in alcohol except at its isoelectric point. It is fairly completely precipitated from water solutions by one-half saturation ammonium sulfate. The substance appears to be an albumose or globulin. Although there appears to be considerable uniformity in the relation between activity and weight of substance, in various purified preparations

made in different ways, the identity of the active substance cannot be regarded as established. The possibility remains that the protein described contains the active material as admixture.

The method of preparation of solutions suitable for experimental work which we have found the most satisfactory is briefly as follows:

For each kilo of finely ground fresh pancreas hash add about 40 cc. of 10 N H_2SO_4 (or 40 cc. concentrated HCl), 1,200 cc. of 95 per cent alcohol, and 300 cc. of water. Stir well at intervals during 4 to 12 hours, at room temperature. Strain through cloth and press out liquid as completely as possible. Reextract hash with a liter or more of 60 per cent alcohol, and again strain and press out liquid. To the combined very turbid liquid add NaOH to faint acid reaction to litmus. Filter through paper, and evaporate filtrate placed in shallow trays in a warm air current (temperature of solution 20 to 30°C.) until all odor of alcohol is gone, or until the volume is reduced to one-quarter to one-tenth the original volume. Transfer the liquid without filtering to a separatory funnel, rinsing the dishes with small amounts of water. Acidify slightly with HCl or H_2SO_4, add 40 gm. of solid $(NH_4)_2SO_4$ for each 100 cc. of solution and shake until dissolved. On standing a few hours the flocculent precipitate first formed rises to the surface and coagulates to a compact layer. Separate the lower liquid as completely as possible, leaving the precipitate in the funnel to drain. When all possible liquid has drained off, add to funnel 75 per cent alcohol (about 50 cc. for each kilo of pancreas hash used), which dissolves the insulin. Rinse the solution and insoluble residue into a centrifuge tube with small amounts of 60 per cent alcohol, and centrifugate. Pipette off the clear alcohol solution and add 8 to 10 volumes of 95 per cent or absolute alcohol. Adjust the reaction of the mixture to C_H 5 to 6 and filter after standing some hours. The precipitate is dissolved in water with the addition of small amounts of either acid or alkali. We ordinarily obtain one "1 kg. rabbit unit" from each 2 gm. of pancreas hash used. Occasionally a better yield has been obtained. For use in human diabetes the material should be purified by means of repeated precipitation at the isoelectric point, and by precipitation by ammonium sulfate, and by alcohol.

NITROGEN DISTRIBUTION IN THE BLOOD AND URINE OF THE ALLIGATOR.

By ALEITA HOPPING and ERNEST L. SCOTT.

(*From the Department of Physiology, Columbia University, New York.*)

Unlike birds and some reptiles, alligators excrete very little uric acid. Blood of the alligator contains from 1.8 to 2.6 mg. of uric acid per 100 cc. of blood, about the same value for human blood. In the urine, uric acid represents from 10 to 20 per cent of the total nitrogen. Uric acid is evidently not the main end-product of nitrogen metabolism in these reptiles. In the urine, ammonia varies from 53 to 80 per cent of the total nitrogen. In one alligator the total nitrogen was 215 mg., the ammonia nitrogen, 171 mg. (79 per cent), urea nitrogen, 7 mg. (3 per cent), and uric acid nitrogen, 40 mg. (18 per cent), per 100 cc. After sterile catheterization, the amount of urea nitrogen gradually increases. In the alligator the cloacal chamber is separated by a sphincter from the intestine and fulfills the function of a bladder. It is possible that decomposition of nitrogenous waste products may occur here. The nitrogen distribution in the blood is being studied and may throw light on this question.

DYE-PROTEIN AGGREGATES.

I. CONGO FIBRIN.

By L. F. SHACKELL.

(*From the Department of Pharmacology and Therapeutics, College of Medicine, University of Illinois, Chicago.*)

A special preparation of Congo fibrin was made by adding an excess of Congo red to a clear hot solution of washed fibrin in 0.1 N NaOH. Neutralization produced a precipitate of dyed *m*-protein. This was coagulated by boiling while in suspension in a saturated solution of NaCl. The excess of dye was removed by repeated washing with boiling distilled water. The Congo fibrin was dried *in vacuo* over sulfuric acid, pulverized, and passed through a 100 mesh sieve.

A suspension of this Congo fibrin in glycerol has yielded no free Congo red to the glycerol in 2 years. A suspension in distilled

water yields up no color in a month when kept at 10°C. The material is rapidly and completely digestible by pepsin in acid solution. The individual particles range in size from 0.01 to 0.25 mm.

Portions of the glycerol suspension have been fractionated by centrifugation so that the range of size of particles in each fraction was fairly small. The velocity of peptic digestion of the largest particles as compared with that of the smallest was much faster than would be expected if digestion were to take place only at the surfaces of the particles. The velocity of digestion of the original suspension was strikingly similar—except in its initial stage—to that of a monomolecular reaction. The graph of the process is, however, simply a skew variation curve. The view is advanced that the monomolecular formula can be applied to protein hydrolyses only by neglecting the details of the earlier stages. A skew variation curve, on the other hand, may mean simply the rapid digestion of numbers of small colloidal particles accompanied by a more leisurely degradation of the larger ones.

THE EFFECT OF INJECTIONS OF VARIOUS SUBSTANCES UPON THE BLOOD COMPOSITION OF THE TORTUGAS CRAWFISH PANULIRUS ARGUS.

By SERGIUS MORGULIS.

(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha.)

The study of the non-protein components of arthropod blood has led to the conclusion that the composition varies rapidly with the changes in the nutritive condition of the animal. A series of experiments was performed with the large crawfish, *Panulirus*, from which blood can be drawn several times without causing the animal any apparent injury. Four substances were studied: glucose, sucrose, urea, and ammonium sulfate. These were dissolved in sea water and injected deep into the thoracic muscles. Blood samples were obtained from a slit in a caudal fin, and were taken prior to the injection and at various times afterwards. Ammonium sulfate was found rather toxic (effect of acidity?) but 0.5 gm. could be tolerated by the animal. This substance gets into the blood almost at once; it has been recovered

about ½ hour after the injection. It is, however, very rapidly excreted and 2 hours later it is completely eliminated and the total non-protein N of the blood is usually diminished as compared with the original content. The behavior towards urea injections is quite different. The largest dose given at one time was 1 gm., but there is good reason to believe that much greater quantities might be given without causing any toxic effects. Striking changes in the blood follow such an injection. A great increase in the non-protein N and in the urea N of the blood occurs, reaching a maximum in about 2 hours. At that time an increase by as much as 70 mg. was sometimes found. The N content of the blood filtrates then commenced to diminish and the original non-protein N level was reached in 12 to 14 hours after the injection. The injection of the urea was always associated with an increase in the uric acid content of the blood. The changes in the uric acid content ran more or less parallel to the changes in the N. Working with crawfish which have been already 1 or 2 days in captivity the change in the uric acid content was particularly striking as the control sample of blood was usually free, or practically so, from uric acid and there was no color, or only the faintest tinge of blue, produced, but the samples obtained 2 to 3 hours after the injection gave intense color reactions. Glucose was injected in quantities ranging from 0.4 to 0.8 gm. The quantity was limited entirely by considerations of convenience of analysis and not by the tolerance which is undoubtedly much greater than 0.8 gm. Temporary hyperglycemia is produced by these injections, and 2 hours after an injection increases in the blood sugar of 155 to 175 mg. were found. The blood sugar curve after reaching a maximum, begins to slope off again and the original sugar level is usually reestablished in 8 to 12 hours. Very interesting changes follow an injection of sucrose. This was injected in doses of 0.4 to 0.5 gm. Within less than 1 hour after the injection the reducing sugar in the blood increases in amount. The blood is also loaded with sucrose as can be shown by the very great increase in hydrolyzable sugar in the blood filtrates. It seems from the experimental data that after the 1st hour the levels of reducing sugar and of hydrolyzable sugar in the blood behave reciprocally. In one crawfish this was followed for 93 hours after the injection. The animal remained

hyperglycemic until almost the very end of this experiment. After 93 hours the original sugar level had been reestablished though there was still 8 mg. of hydrolyzable sugar per 100 cc. of blood.

A STUDY OF THE BLOOD OF THE CRAWFISH PANULIRUS ARGUS WITH SPECIAL REFERENCE TO THE ABSENCE OF CREATININE IN ARTHROPOD BLOOD.

By SERGIUS MORGULIS.

(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha.)

The non-protein constituents of the crawfish blood were studied by the Folin-Wu blood analysis procedure, except for the uric acid determinations which were made according to Benedict's new method. The findings on this arthropod from Tortugas agree well with the results already reported here previously[17] on the blood of several arthropods from Woods Hole. In a large number of animals examined immediately after capture the following variations were found:

	Per 100 cc. of blood.			
	Sugar.	Non-protein N.	Urea N.	Uric acid.
	mg.	*mg.*	*mg.*	*mg.*
Maximum	71	29	11	2.0
Minimum	19	15	6	0.3

Owing to the fact that these crawfish could be bled repeatedly, the question of the influence of the nutritive condition of the animal upon the non-protein composition of the blood could be studied on the same animal. In one instance the changes were observed for 44 hours after the animal had been brought to the laboratory and the results were as follows: the sugar content at the end of 24 and 44 hours changed from the original level of 71 to 18 and 13 mg., respectively; the non-protein N changed from 16 to 14, then to 10 mg.; the uric acid content from 0.7 mg. diminished to 0.3 mg. at the end of 24 hours, and was entirely absent after 44

[17] Morgulis, S., *J. Biol. Chem.*, 1922, l, p. lii.

hours. The complete disappearance of the uric acid from the blood has often been noted to occur in a much shorter time.

The blood filtrates were studied especially with the view of determining if they contain any creatinine. The blood of *Panulirus* at no time showed any trace of this substance and the color developed by the blood filtrates could be read against a blank containing water provided the colors were matched within 15 minutes. The same was also observed in the case of several other arthropods. References in the literature to creatinine in arthropod blood notwithstanding, it can be stated definitely and with the support of an extensive number of analyses that creatinine is never present in the blood of arthropods.

THE ISOLATION OF BILIVERDIN FROM BILE.

By WILLIAM M. BARRY and VICTOR E. LEVINE.

(From the Department of Biological Chemistry, School of Medicine, Creighton University, Omaha.)

To each liter of ox bile are added 50 cc. of 10 per cent HCl. The bile is then placed in a large beaker, left uncovered, and exposed to sunlight until dark green. The acid precipitates the mucin-like substances and nucleo-albumins. The sunlight catalyzes the oxidation of bilirubin to biliverdin. The bile is then carefully decanted, warmed, and extracted with chloroform to remove the cholesterol. It is then extracted with ether until free from ether- and chloroform-soluble substances. To the liquid remaining after this treatment are added 50 cc. of 10 per cent HCl, which precipitates the pigment and the bile acids. The supernatant liquid is drawn off and rejected.

The viscous portion containing the pigment is warmed on a water bath until all the odor of chloroform has disappeared and is then made up to five times its volume with 95 per cent ethyl alcohol. This solution is then diluted with one and a half times its volume of distilled water. The pigment which settles out is washed repeatedly with distilled water, dried, and then washed with ether and again dried.

The pigment prepared thus is an amorphous green solid, insoluble in water, ether, and chloroform, but soluble in glacial acetic acid and in 95 per cent alcohol. It is found to be free from

iron, sulfur (absence of the green pigment, choleprasin), fat, cholesterol, lecithin, and bile salts. The yield is about 2.5 gm. per liter of bile.

THE OCCURRENCE OF KETONE BODIES IN THE URINE OF NORMAL RABBITS IN A CONDITION OF HYPOGLYCEMIA FOLLOWING THE ADMINISTRATION OF INSULIN—A CONDITION OF ACUTE ACIDOSIS EXPERIMENTALLY PRODUCED.

By J. B. COLLIP.

(From the Department of Biochemistry, University of Alberta, Edmonton, Canada.)

The causation of the convulsive state in normal rabbits rendered hypoglycemic by insulin administration—a condition first noted by Collip and Macleod while collaborating with Banting and Best—has been the subject of much study. It occurred to the writer that the glycogen-forming activity of the injected insulin might by lowering the sugar tension on the blood actually jeopardize the oxidation of glucose in the tissue even though a certain amount of sugar did still exist in the blood stream. If such were the case ketone bodies would undoubtedly be formed and the animal would manifest other signs of an acute acidosis.

The urine was therefore obtained from rabbits immediately following the convulsive state induced by insulin, and in every instance a decided positive Rothera test was obtained. The ferric chloride test was not entirely satisfactory as a green precipitate was as a rule formed at first. On filtering, however, a port wine color was usually manifested in the filtrate. Later the urine was expressed from the bladder of rabbits after they had been injected with insulin, but before the blood sugar level had been reduced to the critical level of 0.045 per cent. A positive Rothera test was obtained in animals with blood sugar levels ranging from 0.065 to 0.045 per cent. Quantitative estimation of ketone bodies by the Van Slyke method indicated from 50 to 100 mg. per 100 cc. in samples obtained after the convulsive state had become manifested.

It has also been noted in some cases that as the level of blood sugar falls the CO_2-combining power of the blood falls progressively. In the convulsive state this factor may be as low as 28. If sugar

be administered and the animal allowed to recover, the CO_2-combining power of the blood returns to approximately the original level within a day or two, and the urine also becomes free from ketone bodies. In other instances, however, little or no reduction in the CO_2-combining power of the blood was observed to occur.

These findings are of the greatest interest in that it would appear that while we have in insulin a substance which will correct all the acidotic signs both in experimental animals rendered diabetic, and in patients suffering from diabetes (if the dosage is adequate), it will, nevertheless, produce many of the cardinal symptoms of acidosis when administered to the normal animal in amount sufficient to produce a hypoglycemia of a marked degree.

THE DEMONSTRATION OF AN INSULIN-LIKE SUBSTANCE IN THE TISSUES OF THE CLAM (MYA ARENARIA).

By J. B. COLLIP.

(From the Department of Biochemistry, University of Alberta, Edmonton, Canada.)

When it was shown by the writer in collaboration with Banting, Best, and Macleod that the administration of insulin to a diabetic dog enabled the liver to store glycogen in large amount (25.6 per cent was observed in one instance), it was predicted that wherever glycogen occurs there would be insulin not far distant. The clam was chosen for investigation on account of its high glycogen content, over 11 per cent of glycogen having been recently found in the wet tissue of *Mya arenaria* by Collip.[18]

The method developed by Collip for the isolation of insulin in semipure form from the pancreas of the ox was applied with success to clam tissue. In one experiment an extract so prepared produced typical convulsions in a normal rabbit in 6 hours. The blood sugar at this time was 0.045 per cent and the convulsions were relieved by the subcutaneous injection of dextrose.

Latterly, it has been found that extracts made from clam tissue by a modified process manifest delayed action when administered to normal rabbits. Hypoglycemia of a marked degree is not manifested until the 2nd or 3rd day following the administration of the extract, whereas in certain instances an actual hyperglycemia may be noted for a few hours following the injection.

[18] Collip, J. B., *J. Biol. Chem.*, 1921, xlix, 297.

THE ORIGINAL METHOD AS USED FOR THE ISOLATION OF INSULIN IN SEMIPURE FORM FOR THE TREATMENT OF THE FIRST CLINICAL CASES.

By J. B. COLLIP.

(From the Department of Biochemistry, University of Alberta, Edmonton, Canada.)

The method applied in the preparation of the first insulin used in the treatment of clinical cases was developed by the writer during December and January last. In the critical first few weeks of clinical trial of insulin the preparation of the extract was carried out exclusively by the writer.

This method which is outlined below yielded excellent results at first when small quantities of material were being used; but as the "plant" was gradually enlarged the yield of potent material became less and less. Various modifications of the original method have since been made but it is of interest to note that the general principles developed in the first instance still apply in any modified process. It is now possible by a variety of ways to produce extracts containing the potent principle which are practically protein-, lipoid-, and salt-free. Some of these methods will be described in a subsequent communication. The original method was as follows:

To a small volume of 95 per cent ethyl alcohol, freshly minced pancreas was added in equal amount. The mixture was allowed to stand for a few hours with occasional shaking. It was then strained through cheese-cloth and the liquid portion at once filtered. The filtrate was treated with 2 volumes of 95 per cent ethyl alcohol. It was found by this treatment that the major part of the protein was removed while the active principle remained in alcoholic solution.

After allowing some hours for the protein precipitation to be effected the mixture was filtered and the filtrate concentrated to small bulk by distillation *in vacuo* at a low temperature (18 to 30°C.). The lipoid substances were then removed by twice extracting with sulfuric ether in a separating funnel and the watery solution was returned to the vacuum still where it was further concentrated till it was of a pasty consistency. 80 per cent ethyl alcohol was then added and the mixture centrifuged.

After centrifuging, four distinct layers were manifested in the tube. The uppermost was perfectly clear and consisted of alcohol holding all the active principle in solution. Below this, in order, were a flocculent layer of protein, a second clear watery layer saturated with salt, and a lowermost layer consisting of crystals of salt. The alcohol layer was removed by means of a pipette and was at once delivered into several volumes of 95 per cent alcohol, or better, of absolute alcohol. It was found that this final treatment with alcohol of high grade caused the precipitation of the active principle along with adherent substances. Some hours after this final precipitation the precipitate was caught on a Buchner funnel, dissolved in distilled water, and then concentrated to the desired degree by use of the vacuum still. It was then passed through a Berkefeld filter, sterility tests were made, and the final product was delivered to the clinic.

The essential points relating to the extract prepared as outlined above are: (1) It contains only a minimum of protein. (2) It is practically salt-free and can readily be made isotonic. (3) It is lipoid-free. (4) It is almost free from alcohol-soluble constituents. (5) It can be administered subcutaneously without fear of any local reaction.

Note.—This method was developed by the writer while he was attached to the Department of Pathological Chemistry, University of Toronto.

STUDIES ON BY-PRODUCT YEAST.

By JOSEPH S. HEPBURN.

(*From the Constantine Hering Laboratory, Hahnemann Medical College, Philadelphia.*)

Thoroughbred albino rats have been fed a diet consisting of purified foods to which dried yeast has been added as the sole source of the water-soluble B vitamine. Three grades of by-product yeast have been used: (1) bottom yeast, derived from manufacture of industrial alcohol from waste molasses; (2) brewery yeast; and (3) ale yeast.

All three grades were dried by the Wittemann process. The rats have grown and reproduced, and the offspring have likewise

gained in weight. When the dried yeast from the industrial alcohol plant was included in the ration fed to immature chickens, they showed excellent gains in weight.

THE TITER VALUE OF CHICKEN FAT.

By JOSEPH S. HEPBURN.

(From the Constantine Hering Laboratory, Hahnemann Medical College, Philadelphia.)

The titer test was applied to twenty composite samples of abdominal fat from the common fowl, *Gallus domesticus*. The titer of the isolated insoluble fatty acids ranged between 32.50 and 37.60°C., the average for the twenty samples being 34.94°C.

A NOTE ON THE INITIAL ACIDOSIS OCCURRING WITH ANESTHESIA.

By GLENN E. CULLEN and J. HAROLD AUSTIN.

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia.)

In attempting to determine the mechanism of the marked acidosis which occurs with ether anesthesia and which is indicated by increase in the hydrion concentration, and decrease in the alkaline reserve of the blood, studies have been carried out with inhalation of other anesthetics as well as with other gases.

In previous studies we have found the acidosis existing ½ hour or more after commencement of the anesthesia.

In our present investigation we have made repeated observations during the first few minutes after commencing administration of the anesthetic and have found, within 5 minutes, an increase in the hydrion concentration and a decrease in alkaline reserve comparable with that present at the end of an hour.

Moreover, we have observed this initial acidosis after inhalation not only of the anesthetics, ether, chloroform, and nitrous oxide, but also of formaldehyde, pure nitrogen, and pure oxygen.

THE INFLUENCE OF ALKALI THERAPY ON KETOSIS IN DIABETES MELLITUS.

By HERMAN O. MOSENTHAL and JOHN A. KILLIAN.

(From the Departments of Medicine and of Biochemistry, New York Post-Graduate Medical School and Hospital, New York.)

Six patients confined in the Metabolic Ward were placed upon a standard diet comprising about 30 gm. of carbohydrate, 130 gm. of fat, and 40 gm. of protein, giving 1,400 to 1,500 calories. A preliminary period of 7 to 10 days permitted an adjustment of the patient's metabolism to this diet. During the experimental period, sodium bicarbonate was given by mouth in doses of 30 to 45 gm. per day until it was felt that the maximum effect of the alkali had been obtained. The after period was extended until a full recovery from the influence of the alkali was noted. In some instances two experimental periods were employed. Observations were made upon the sugar, acetone bodies, and carbon dioxide-combining power of the blood; and the total nitrogen, sugar acetone bodies, and reaction of the urine. Van Slyke's method was used for the determination of the acetone bodies. It was noted that the administration of the alkali produced a decrease in the acetone bodies of the blood associated with a rise in the carbon dioxide-combining power, and a drop in the concentration of sugar. At the beginning of the period of alkali therapy there was an initial rise in the acetone bodies of the urine but subsequently they fell, to within normal limits in some cases. The sugar excretion in the urine was also markedly decreased. In some instances the effect of the alkali therapy persisted for about 10 days after the sodium bicarbonate had been discontinued.

A SIMPLE METHOD OF ESTIMATING THE SALIVARY UREA.

By HERBERT W. SCHMITZ.*

(From the Department of Biochemistry, New York Post-Graduate Medical School and Hospital, New York.)

Since the concentration of the urea in the saliva is practically equal to that in the blood, the utilization of salivary urea estimations in determining the degree of urea retention has been recom-

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mended in a previous paper.[19] A simple method for estimating the salivary urea is described.

Technique: Into a large test-tube (graduated at 15 and 20 cc.) are introduced 2 cc. of filtered saliva, 2 drops of molecular phosphate, and 1 cc. of a 5 per cent urease solution. The tube is incubated in a beaker of water at 50°C. for 15 minutes. At the end of this time distilled water is added to the 15 cc. mark, 2 cc. of dialyzed iron (Merck's, 5 per cent Fe_2O_3), 1 cc. of a 20 per cent sodium sulfate solution, and distilled water to the 20 cc. mark. After the mixture has been shaken vigorously, it is poured upon a folded filter. 10 cc. of the filtrate (equal to 1 cc. of saliva) are now pipetted into a graduated cylinder and 5 to 10 cc. of dilute Nessler's solution, dependent on the content of nitrogen, are dumped in at once while the solution in the cylinder is rotated until the maximum color development is obtained. If a large amount of urea is present, sufficient distilled water is added until the color of the unknown approximates the color intensity of the standard. The colors are matched immediately in the colorimeter.

The results obtained with this method agree very favorably with those obtained with the aeration procedure.

A PHYSICOCHEMICAL METHOD OF CHARACTERIZING PROTEINS. IV.

By EDWIN JOSEPH COHN.

(From the Laboratory of Physical Chemistry, Harvard Medical School, Boston.)

In previous reports to this society we have pointed out: (1) that the differences in the solubility of proteins that have led to their classification depend in large part upon their dissociation at their isoelectric points; (2) that the solubility of each protein that we have purified of its soluble compounds was constant at its isoelectric point; and (3) that this solubility represented the sum of the concentrations of the undissociated protein molecule and of the ions into which it dissociates.

By studying solubility in systems containing a protein and small amounts of its soluble compounds, we have succeeded in

[19] Schmitz, H. W., *J. Lab. and Clin. Med.*, 1922, viii, 78.

estimating the degree of dissociation of certain proteins at their isoelectric points. The methods that we have employed have been developed from the fundamental equations for the dissociation of amphoteric electrolytes. Their consideration has also yielded a graphical method of analyzing the solubilities of

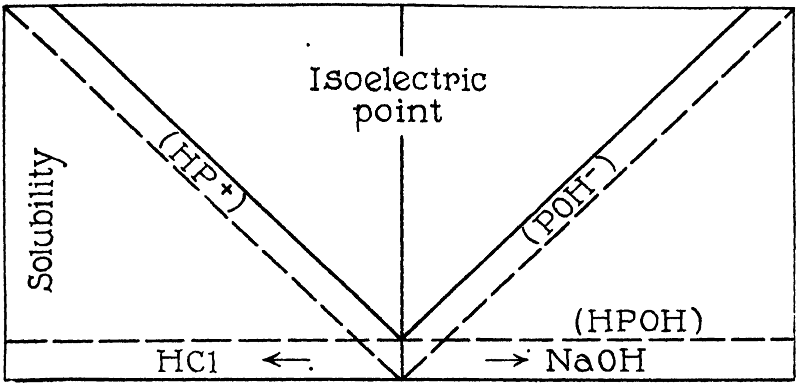

FIG. 1.

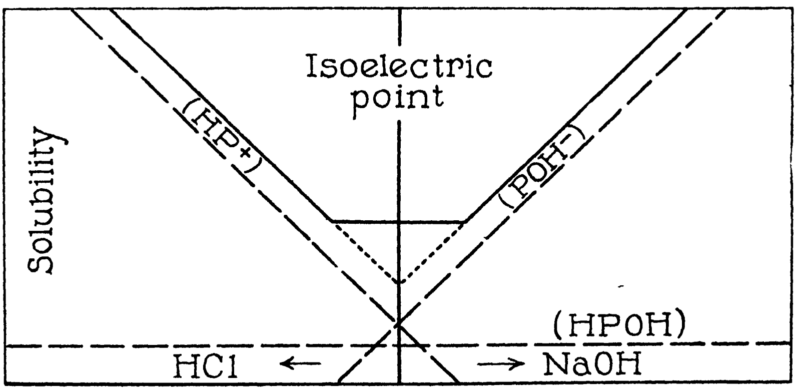

FIG. 2.

proteins over a small range—where they may be considered as dissociating as monovalent acids and bases—in the neighborhood of their isoelectric points.

The solubility relations of two hypothetical proteins are represented in Figs. 1 and 2. The ordinates designate concentrations

of the undissociated molecule (HPOH) and of the basic (HP^+) and the acidic (POH^-) ions; the abscissæ the excess of acid, or of base. The protein in Fig. 1 is not appreciably dissociated at its isoelectric point. The concentration of ions formed by its dissociation is, therefore, proportional to the acid or base with which it combines. A straight line represents the constant concentration of the undissociated molecule. Irrespective of the value ascribed to the latter, the "curve" describing the solubility of such a protein, therefore, consists of two straight lines, diverging from the isoelectric point.

Curves describing the solubility of proteins have, however, generally passed through rounded minima in the neighborhood of the isoelectric points. This suggests that the proteins were in part dissociated. In Fig. 2 the hypothetical protein combines with acids and bases in the same ratio as the protein in Fig. 1. The protein is, however, a stronger acid and a stronger base, and is, therefore, partly dissociated at its isoelectric point. The value of the ordinate at the point of intersection of the (HP^+) and the (POH^-) lines is a measure of the concentration of these ions at the isoelectric point. If the concentration of the undissociated molecule is added to the concentration of these ions, the solubility of the protein in the system is obtained. This solubility passes through a wide minimum at the isoelectric point. The "solubility curve" becomes a straight line, however, at such a distance from the point that the concentration of one of the ions becomes negligible in comparison with the other.

Our experimental determinations of the solubility of casein in systems containing small amounts of sodium hydroxide fall on such a straight line. Extension of this straight line to its point of intersection with the isoelectric line yields the concentration of the undissociated molecule and the casein anion.

$$(\mathrm{HPOH}) + (\mathrm{POH}^-) = 0.085 \pm 0.005 \text{ gm. per liter at } 25^\circ\mathrm{C}.$$

But we have previously reported the solubility of isoelectric casein:

$$(\mathrm{HPOH}) + (\mathrm{POH}^-) + (\mathrm{HP}^+) = 0.11 \pm 0.005 \text{ gm. per liter at } 25^\circ\mathrm{C}.$$

By difference (HP^+) equals 0.025 ± 0.01, and since at the isoelectric point (HP^+) = (POH^-), the latter also equals 0.025; (HPOH) equals 0.06; and the degree of dissociation:

$$(HP^+)\ (POH^-) : (HPOH)^2 = Ka\ Kb : Kw$$

must lie between 0.1 and 0.4.

CONCERNING THE NATURE OF COHESION.

By A. P. MATHEWS.

THE METABOLISM OF CREATINE.

By S. R. BENEDICT and EMIL OSTERBERG.

THE RELATIONSHIP BETWEEN STRUCTURE AND CHEMICAL PROPERTIES OF SOME SUBSTANCES RELATED TO THYROXIN.

By E. C. KENDALL.

RATE OF AMMONIA LIBERATION IN TRYPTIC DIGESTION OF PROTEIN.

By ANDREW HUNTER and R. G. SMITH.

ON NORMAL BLOOD SUGAR VALUES IN MAN AND ANIMALS

By W. S. McELLROY and H. O. POLLOCK.

THE PROTEIN REQUIREMENTS IN DIABETES.

By A. I. RINGER, S. BLOOM, and S. JACOBSON.

MINERAL DEFICIENCIES OF MILK AS SHOWN BY GROWTH AND FERTILITY OF WHITE RATS.

By AMY L. DANIELS and MARY K. HUTTON.

THE EFFECT OF HEAT TREATMENT OF MILK FEEDINGS ON THE MINERAL METABOLISM OF INFANTS.

By AMY L. DANIELS and GENEVIEVE STEARNS.

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